University of Alberta

Immunology of Decellularized Allograft Tissue Used in Congenital Cardiac Surgery

by

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DEDICATION

This text is dedicated to my wife Cori-Lynn who has supported me tirelessly throughout this project and to The Boys whose zest for life brings cheer to even the most arduous days in the laboratory.

ABSTRACT

Cryopreserved allograft tissue is used extensively in congenital cardiac surgery, and to a lesser extent in adult cardiac surgery (as valves and patches). This tissue is very immunogenic, stimulating a profound cellular immune response which has been correlated with the failure of this tissue, especially valves. Less clear was the humoral immune response to this tissue. In a prospective cohort study we clearly demonstrated that cryopreserved pulmonary artery patches used in repair of hypoplastic left heart syndrome (HLHS) stimulate a profound donor-specific humoral immune response with panel reactive antibodies (PRA) approaching 100%. This sensitization is concerning as an unknown proportion of these children may require cardiac transplantation in the future and it has been well documented that the presence of preformed anti-HLA antibodies negatively impacts the outcomes of cardiac transplantation.

It has been shown that the stimulus of the alloreactive immune response is the cellular elements of the allograft tissue and thus decellularization was hypothesized as a method to reduce the immunogenicity of these tissues. We compared a number of different decellularization techniques. A combination of hypotonic/ hypertonic buffers, mild detergent (Triton X-100), and washout in PBS were found to be the most effective method to remove antigenic cellular elements while maintaining the integrity of the extracellular matrix. In a rat allograft model we then demonstrated that decellularization abrogates both the cellular and humoral immune response to allograft tissue. Additional in vitro testing using porcine tissue confirmed preservation of the biomechanical properties of these tissues.

A systemic approach with the immunomodulatory agent intravenous immunoglobulin (IVIG) was also investigated. In a pilot trial of infants with HLHS, IVIG was found to be ineffective in preventing sensitization to allograft tissue used at the time of surgery despite previous reports of efficacy of desensitizing individuals with elevated PRAs awaiting organ transplantation.

In summary, cryopreserved allograft tissue used in congenital cardiac surgery is highly immunogenic, stimulating significant cellular and humoral immune responses. By removing the immunogenic cellular elements of this tissue, we clearly demonstrated abrogation of the alloreactive immune response in a rodent model. Abrogation of the cellular immune response may prolong the durability of valve allografts, thus decreasing the need for repeat operations for allograft valve failure. Abrogation of the humoral immune response is especially relevant for those with congenital heart disease that require allograft tissue as part of their surgical repair as some may require future heart transplantation. These findings could have a significant impact on the surgical management of children with congenital heart disease. Confirmation of preserved biomechanical properties and reduced immunogenicity will need to be confirmed in a larger animal model.

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DISCLAIMER

This document has been formatted as a "paper based" thesis in accordance with the University of Alberta Faculty of Graduate Studies guidelines. Thus, each chapter (exclusive of Introduction and Discussion chapters) represents manuscripts which have either been submitted for publication, accepted for publication, or published. As such, some redundancy in the Introductory and Discussion sections of individual experimental chapters exists.

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LIST OF ABBREVIATIONS

^{1}H-NMR	proton nuclear magnetic resonance
ANOVA	analysis of variance
CDC	complement-dependent cytotoxicity
CPB	cardiopulmonary bypass
CREG	cross-reactive group
DMSO	dimethyl sulfoxide
DNASE	deoxyribonuclease
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FCXM	flow cytometric crossmatch
FFP	fresh frozen plasma
FITC	fluorescein isothiocyanate
FLOWPRA	flow cytometric panel reactive antibody
GAG	glycosaminoglycan
H&E	hematoxylin and eosin
HBSS	Hank's balanced salt solution
HLA	human leukocyte antigen
HLHS	hypoplastic left heart syndrome
IVIG	intravenous immunoglobulin
MHC	major histocompatability complex

MMF	mycophenolate mofetil
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PRA	panel reactive antibody
PRBC	packed red blood cells
RNASE	ribonuclease
SD	standard deviation
SEM	standard error of the mean
SEM	scanning electron microscopy
TCA	total circulatory arrest
TEM	transmission electron microscopy
XC	cross clamp

I

INTRODUCTION

RATIONALE

Valve replacement has become increasingly common with over 4000 replacement operations performed annually in Canada. Existing replacement options include mechanical valves which require lifelong anticoagulation and bioprosthetic valves (porcine) which deteriorate with time. A third option for valve replacement is cryopreserved allograft tissue. This valve has many of the essential characteristics of the ideal replacement valve including absence of thrombogenicity, resistance to infection, and excellent hemodynamics. However, like bioprosthetic valves, allograft valves have limited (10-15 year) durability. While used infrequently in adult cardiac surgery, allograft valves are essential for many of the complex surgical reconstructions of congenital cardiac disease in children in whom they are used whole (as valved conduits) or in part (as patches). In children, however, durability is extremely limited (less than 5 years) after which valved tissues require surgical replacement.

Native valves are living structures that continually remodel their structural proteins. Allograft valves do contain living cells, at least initially; however, these cells are potentially immunogenic. Clinical studies and animal models by our group and others have provided strong evidence that allograft tissues elicit an intense cellular immune response, supporting the concept that that immune factors are likely important in the long-term durability of allograft heart valves. Additional evidence exists that alloreactive immune response is stimulated by the cellular elements in the allograft tissue. Less clear,

but equally concerning to the role of the cellular immune response in allograft valve failure, is the potential for these tissues to stimulate the generation of donor-specific antibodies. These antibodies may affect valve durability and, more importantly, may complicate future heart transplantation, if ever required.

There are two options to attenuate this alloreactive response: alter the host (e.g. immunosuppression) or alter the valve. Currently available immunosuppression is not acceptable for many valve candidates, especially young children, due to toxicity. Altering the valve would require a tissue-engineering approach (Figure I-1). **It is hypothesized that removal of the cellular elements (i.e. decellularization) will abrogate the alloreactive immune response. This, in turn, will 1) prevent immune mediated damage to the valve and 2) improve valve durability, and 3) prevent the generation of donor-specific antibodies.**

The ultimate long-term goal is to create a nonimmunogenic acellular matrix capable of repopulation by host cells, cells that would also be nonimmunogenic and capable of ongoing repair and remodeling of the valve. This would have enormous implications for the survival and quality of life of all allograft valve recipients, especially children.

ALLOGRAFT VALVES

Despite forty years experience the search for the ideal cardiac valve replacement continues. The essential characteristics of the ideal valve replacement include durability, absence of thrombogenicity, resistance to infection, normal hemodynamics, and the capacity to remodel, repair, and possibly even grow.[1] Current valve substitutes include

bioprosthetic (porcine/bovine pericardium) valves, mechanical valves, and allografts (i.e. homografts). Existing mechanical heart valves require lifelong anticoagulation with coumadin with all of its lifestyle-limiting and bleeding-related complications.[2,3] Bioprosthetic valves deteriorate with time in adults, and more rapidly in children.[4,5] Both are prone to infection and thromboembolic events.

Allograft valves were introduced for replacement of diseased cardiac valves in 1962[6] and for reconstruction of the right ventricular outflow tract in 1966.[7] Numerous studies have demonstrated the advantages of the allograft including superior hemodynamics, freedom from anticoagulation, minimal thromboembolic complications, resistance to infection, and good long-term durability.[8-11] Furthermore, without the use of allograft conduits many of the complex reconstructive congenital surgeries would not be possible in children who rapidly degrade bioprosthetic (xenograft) valves and for whom anticoagulation is difficult. However, current allografts ultimately fail with a freedom from reoperation for all causes of 69% at 15 years.[12] The rate of failure in children less than 3 years of age is much higher with a reoperation rate of 60% (after 2.3 years of follow-up) and a mean interval for replacement of 1.9 years after the original operation.[13] Accordingly, there is strong incentive to develop a better valve substitute: this could decrease or prevent the need for repeat valve replacements, again, especially in young children for whom it is not uncommon to undergo three or four surgeries throughout their childhood.

ALLOGRAFT FAILURE

Originally, allograft valves were prepared using harsh methods including physical techniques (freeze drying, gamma irradiation) [14,15] and chemicals (β-propriolactone).[16] These valves were completely acellular and were associated with unacceptably high rates of valve failure[17-19]. Antibiotic sterilization[20-22] and cryopreservation evolved as methods to preserve pre-implant cellular viability and were associated with improvement of long-term results. The disadvantage of antibiotic storage has been a limited shelf life (Table I-1) and the consequent difficulty in maintaining an adequate selection of these allografts. O'Brien's work refined cryopreservation techniques and tissue-banked cryopreserved allografts are now the primary source of cardiac allograft tissue.[8-11] Like antibiotic storage, cryopreservation seeks to maintain maximum cellular viability in the belief that an intact and viable valve will have the capacity to maintain the extracellular matrix, remodel, and possibly even grow. Viability studies revealed that the cryopreserved valves do indeed have intact interstitial cells at the time of implantation but the endothelium is less well preserved. Clinical follow up has revealed the superiority of the cryopreserved allograft compared to the previous preservation techniques with freedom from reoperation for all causes approaching 69% at 15 years.[12] These results, however, are only slightly better than that of bioprosthetic valves. Fresh (homovital) valves which are used within 48 hours of harvest have also been promoted by a number of groups; cell viability at implantation has been proven but long-term durability is not as good as cryopreserved valves.[23,24]

Technique	Procurement	Warm ischemia time	Sterilization technique	Storage technique	Storage time
Antibiotics	Aseptic	Hours–few days	Moderate-dosage antibiotics	4°C	Hours–days
Cryopreservation	Aseptic	<6 hours	Low-dosage antibiotics	−72°C, −196°C	Months–years
Fresh	Aseptic	<2 hours	Nutrient medium (e.g., I-199)	4°C	<72 hours

Table I-1. Current allograft preservation techniques. (From: Aortic Valvular Allografts and Pulmonary Autografts. In: Edmunds LH, editor. Cardiac Surgery in the Adult. New York: McGraw Hill; 1997. p. 912.)

Despite evidence of viable cells at implantation, however, cryopreserved valves have been found to be either partially or completely acellular at subsequent explantation.[25-27] Koolbergen et al. examined 40 allografts acquired from patients whose grafts had failed for a variety of reasons (stenosis, insufficiency, paravalvular leakage, stenosis with endocarditis, technical failure) at 14 days to 16 years following implantation.[27] Using macroscopy, light and electron microscopy, and immunohistochemistry they identified a significant reduction in cellularity of the valve tissue, loss of trilaminar architecture, absence of endothelial cells, and evidence of low-grade inflammatory response. No convincing evidence of immune mediated injury was found, however, at these delayed explantation times. Identifying an explanation for the progressive loss of cellularity and ultimate failure of allograft heart valves despite improvements in preservation and storage techniques has been the goal of numerous investigators. Potential causes for cellular loss include variables in the harvest, preparation, and preservation of aortic allografts. An immune-mediated process may also be involved and this will be the underlying focus of the remainder of this discussion.

ALLOREACTIVE IMMUNE RESPONSE

Initially, allograft valves were thought to be immunoprivileged and thus no attempts were made to match for HLA type or use immunosuppression. However, using a variety of investigative techniques our group and others have provided evidence that allograft valves do activate alloreactive immune responses. One study paradoxically correlated prolonged allograft retrieval-to-cryopreservation times (>24 hours; P = 0.02) with improved allograft valve durability.[28] The conclusion from this study was that prolonged allograft retrieval-to-cryopreservation times were associated with reduced cellular viability, reduced immunogenicity, less immune-mediated destruction, and improved durability. These findings were in complete contradiction to the aforementioned studies in the previous paragraphs which strived to define optimal cryopreservation parameters in order to preserve cellular viability. A recent follow up study from the same group with a larger sample size reconfirmed these findings.[29] In addition, HLA-DR mismatch was identified as a significant predictor (P = 0.03) of allograft valve failure on multivariate analysis. These studies demonstrate that multiple immunologic features are associated with allograft failure. Others have looked at the systemic immune response in humans and animals. Findings include an increase in donor-specific cytotoxic[30,31] and helper[32] T lymphocyte precursors as well as evidence for a humoral immune response with donor-specific IgG to donor HLA Class I and II antigens[33,34] following allograft implantation. When the specificity of the immune response was assessed in these studies it was found to be specific for the donor graft HLA type.

Pathological studies have compared explanted allograft heart valves and aortic valves of explanted heart transplants.[25,26] Explanted aortic valve allografts revealed

progressive collagen hyalinization, focal calcification, loss of normal structural complexity, and loss of cellularity. On the other hand, aortic valves from explanted heart transplants demonstrated negligible calcification, optimal cusp viability, minimal evidence for acute rejection (even in the setting of myocardial parenchymal rejection or graft atherosclerosis), and preservation of tissue components (collagen and elastic fibers, proteoglycans, and intrinsic nervous ganglia). These findings have been attributed to immunosuppression.

Using a rat model our group has provided strong evidence for an alloreactive immune response causing allograft failure.[35,36] Rat aortic valve allografts were transplanted in to the infrarenal aorta of syngeneic (Lewis to Lewis) and allogeneic (Brown Norway to Lewis) rats. Allogeneic grafts demonstrated early (7 days) T-cell infiltrates followed by leaflet thickening, loss of cellularity, and eventual leaflet destruction by 28 days. Syngeneic grafts showed minimal leaflet infiltration and maintained normal leaflet architecture. Similarly, allografts in T-cell deficient rats and rats that received immunosuppression showed none of the aforementioned abnormalities.[37] Similar findings have been reported by other groups using different inbred rodent strain combinations.[38-41] Taken together, these findings provide evidence for a cellular immune response-mediated mechanism of failure of valve allografts. Less clear, however, is the role of the humoral immune response.

Realizing that there is a donor specific immune response led a number of groups to investigate more closely the effect of donor HLA mismatch and the humoral immune response in allograft recipients. As early as 1994, Hoekstra et al[42] using an in vitro lymphocyte proliferation assay were able to demonstrate stimulation of peripheral blood

monocytes with both fresh and cryopreserved allografts tissue. In this study, HLA-DR matching resulted in a significant reduction (P = 0.006) of monocyte stimulation. Subsequent clinical investigations by the same group[43] confirmed the immunogenic potential of cardiac valve allografts in vivo: they found that panel reactive antibodies (PRA) developed in 78% of 32 recipients of cardiac valve allografts. Smith et al. noted similar findings. In their initial studies they reported a strong donor HLA-specific antibody response with HLA antibodies detected in 56% of recipients of antibiotic-preserved allografts and 100% of homovital (fresh) allograft recipients.[44] Hawkins et al. similarly identified a significant increase in alloreactive antibodies in 24 children receiving cryopreserved allografts: at 3.3 months after operation panel reactive antibodies had risen to 92% (1.9% preoperatively) and Class II antibodies had risen to 70%.[45] Dignan et al., in examining the outcomes of 162 recipients of cryopreserved homografts in which both donor and recipient HLA type were known, identified a correlation (P = 0.04) between HLA class II mismatch and freedom from structural valve deterioration at long-term follow up (>5years).[46] This was one of the first studies to find evidence for an association between allograft dysfunction and an HLA (Class II) mismatch. Using stepwise dilution of plasma and complement-dependent microlymphocytoxicity assays, others have correlated anti-HLA antibody titres and allograft valve failure.[47]

An additional concern of the humoral immune response to allograft tissue is the impact on future organ transplantation, if ever required.[48-52] It has been demonstrated that pretransplant anti-HLA antibodies (i.e. sensitization) measured as the PRA are associated with early development of high grade cellular rejection and increased annual rejection frequency,[53,54] increased graft vasculopathy,[55] and decreased survival.[56,57]

Notably, Jacobs et al. recently reported that in paediatric transplantation (median age 130 days), a pretransplant PRA > 10% was associated with increased thirty-day (25%) and overall (50%) mortality compared with those with a PRA < 10% (8% and 15%, respectively).[57] Moreover, elevated PRAs increase the time on the wait list and complicate perioperative management at the time of transplantation.

The source of immunogenicity is unclear but is thought to arise from the cellular components of the valve. It is documented that endothelial cells express both Class I and II HLA antigens and that the interstitial cells (fibroblasts) express HLA Class I antigens. A number of studies have suggested that endothelial cells are the prime source of immunogenicity.[58,59] Hoekstra et al.[60] performed lymphocyte stimulation assays on pieces of fresh and cryopreserved allografts with human peripheral blood lymphocytes both matched and mismatched for HLA antigens. They found that lymphocyte stimulation to cryopreserved tissue was less than to fresh tissue, suggesting that cryopreservation reduces immunogenicity, possible through a reduction of viable endothelial cells. Lymphocyte stimulation was further and significantly reduced ($p=0.006$) when lymphocytes were matched for HLA-DR, thus supporting a role for HLA-DR mismatch in allograft failure. Batten et al. investigated the role of individual cellular components of the donor valve in stimulating the immune response.[59] The proliferative response of highly purified T cells was used to assess the immunogenicity of cultured valve endothelial and interstitial cells. T cell responses to endothelial cells were detected after interferon gamma treatment but were not detected in interferon gamma-treated interstitial cells, leading this group to conclude that only the endothelial cells were immunogenic. These findings confirmed previous work by the same group in which valve endothelial

and interstitial cells were co-cultured with peripheral blood mononuclear cells and allogeneic T cells.[58] Again, valve endothelial cells but not fibroblasts were found to be capable of stimulating T cells in vitro, including direct stimulation of CD4+ T cells. In the same study, samples of valve tissue which had been incubated for varying periods of time in a standard antibiotic sterilization solution (which is commonly used in the processing of human valve allografts for patient use) were assessed in a similar fashion for immunogenicity. These in vitro studies revealed incubation in antibiotic solution decreased immunogenicity in a time and dose-dependent manner, possibly through reduction in endothelial cell integrity. These findings confirmed the hypothesis of Baskett et al. that prolonged harvest-to-preservation times were associated with reduced immunogenicity and improved allograft valve durability in humans.[28] Simon et al analyzed the expression of cell surface molecules known to be involved in rejection and inflammatory responses using a range of monoclonal antibodies.[61] In addition to identifying a large variety of cell surface molecules involved in rejection, they were able to conclusively identify HLA Class II antigens on cardiac valve endothelium, a previously debated finding. Thus, it can be demonstrated that the endothelial layer appears to be highly immunogenic and may play a vital role in the alloreactive immune response elicited by allografts. Moreover, detailed immunohistochemical assessment of the valve allograft endothelial cells reveal surface markers characteristic of activated endothelial cells (APC) and these cells may play a critical role in activation of a specific immune response.[62] The role of interstitial cells and the extracellular matrix in the alloreactive immune response is less well understood.

As noted above, studies of cryopreserved allografts in humans have demonstrated that. despite initially intact cellular components, there is a progressive loss of cellularity and an associated loss of valve function.[22,63,64] These long-term human studies have also demonstrated a paucity of immune infiltrates, leading some to suggest that allograft failure is not an immunologic based process. This conclusion is made despite the evidence presented in the previous paragraphs. Nevertheless, this conclusion may be partially correct: the long-term durability of allografts may not be directly related to an immunologic response. However, the initial intense inflammatory response may result in loss of interstitial and endothelial cells and a consequent loss of the ability of the valve to regenerate and repair itself. Particularly concerning is the loss of interstitial cells as they are responsible for the maintenance of the amorphous and fibrillar extracellular matrix. Thus, long-term degeneration of the quickly denuded allograft might be due to an increased sensitivity to mechanical stress after early immunologic decellularization. Furthermore, in addition to destruction of donor cells, the lymphocyte infiltrates may cause damage to the allograft extracellular matrix that, once altered, will not support cell adhesion and repopulation by recipient endothelial and interstitial cells.[65]

ATTENUATION OF IMMUNE RESPONSE

There are two options to attenuate this alloreactive response: alter the host (e.g. immunosuppression) or alter the valve. Short courses of immunosuppression have been tried in animal models[37,66] and humans[67] with positive outcomes. Additionally, the beneficial effects of immunosuppression have been demonstrated in explanted heart transplants.[25,26] Immunosuppression in its current form, while effective, is not clinically

acceptable, especially in children due to toxicity and the increased risk of infection.[68-71] Tissue typing and matching for HLA type have been proposed; however, this would require a supply of allografts larger than most institutions currently are able to maintain, a problem that could potentially be overcome by an organ-sharing network.[72]

An alternative and safer[73] immunomodulatory agent is intravenous immunoglobulin (IVIG).[74-76] Experience with kidney and cardiac transplants has demonstrated that IVIG can produce clinically significant and sustained reductions in anti-HLA antibody titres in individuals who have been previously sensitized, in turn allowing for successful transplantation.[77-87] There have been numerous case series and several trials documenting the effectiveness of IVIG in desensitizing individuals with elevated PRAs and awaiting organ transplantation. John et al. reported a 33% reduction ($P < 0.01$) in PRA levels with IVIG with 1 to 3 monthly courses of IVIG (2 g/kg), in turn allowing for earlier cardiac transplantation of highly sensitized individuals.[86] Glotz et al. reported the successful desensitization in 13 of 15 (87%) patients in a pilot trial with 3 monthly courses of 2 g/kg body weight IVIG.[87] These 13 patients underwent immediate kidney transplantation, with loss of only one of the kidneys to rejection. Lastly, the NIH-sponsored IG02 trial randomized 101 adult end-stage renal disease patients with PRA > 50% to IVIG (2 g/kg monthly for 4 months) or placebo.[88] IVIG therapy was associated with a modest improvement in transplantation rates (35% vs. 20%; $P = 0.069$) on intention-to-treat analysis. IVIG was also associated with reduced time to transplantation ($P < 0.05$) and decreased mortality (8% vs. 16%; $P = 0.22$). These findings of increased transplantability occurred despite only a modest reduction in IgG PRA values (absolute reduction < 20%; $P = 0.007$ on repeated measures analysis) and failure to reduce PRA

levels to < 40%. Despite numerous reports of desensitizing individuals with elevated PRAs, there are no reports of preventing sensitization and this requires additional investigation.

Altering the valve, on the other hand, would require a tissue-engineering approach. The goal of tissue engineering would be to create a truly living valve structure that will have a nonthrombogenic endothelial surface and a living interstitium that would be capable of repair and remodeling. Numerous groups have been investigating different tissue-engineering techniques using synthetic, allogeneic, and xenogeneic material. Mayer et al. have been investigating the use of biodegradable synthetic scaffolds (e.g.: polylactic acid, polyglycolic acid, polyglactin, polyhydroxyoctanoate) on to which they seed individual (interstitial and endothelial) cellular components.[89-96] This approach has also yielded promising results with histologic examination revealing organized viable tissue and biochemical studies revealing extracellular matrix protein production six months after implantation in an ovine model, but requires an inconvenient in vitro seeding of the valve with autologous cells. Questions that have yet to be answered by this group include what is the best scaffolding material? what types of cells should be used to repopulate the matrix? and whether or not progenitor or stem cells circulating in the blood can repopulate the matrix in vivo? or must the cells be cultured on to the scaffolds in vitro? Furthermore, the long term durability of these constructs is not known as is the ability to translate the current animal studies to human valve constructs.

An alternative scaffold would be of allogeneic origin, consisting of a completely decellularized matrix. As mentioned above, the endothelial and interstitial cells are believed to be the immunogenic stimulus for the alloreactive immune response leading to

failure of the allograft. It is felt that because the interstitial matrix proteins are conserved within a species they should not be immunogenic when transplanted as allografts.[97] It is therefore believed that a decellularized allograft will not only be nonimmunogenic but also capable of repopulation by host interstitial and endothelial cells, in turn restoring the regenerative capabilities of the allografts, thus improving its durability. Numerous techniques for decellularization of allografts have been investigated by a number of investigators and include detergents,[98,99] enzymes,[100,101] hypotonic solutions,[102] or some combination thereof. Regardless of the decellularization process used the principles remain the same: 1) the process should not grossly disrupt the anatomy of the transplant tissue, substantially alter the biochemical properties of its structural elements, or produce a cytotoxic environment that mitigates against subsequent repopulation of the matrix with recipient endothelial and interstitial cells and 2) it should significantly reduce the immunogenicity of the allograft tissue. In order to fulfill the latter goal, it will be essential that decellularization protocols not only kill donor allograft cells, but effectively remove the cells and their major (i.e. membrane-bound HLA molecules) and minor antigens.

Detergent-based techniques using nonanionic detergents such as Triton X-100 disrupt cell membranes and aid in the removal of cellular debris from the tissue. However, care must be taken to eliminate any residual detergent so as to prevent interference with the subsequent repopulation of the tissue matrix with viable recipient cells. Courtman et al. have reported on the use of a detergent-based (Triton X-100) decellularization process in a variety of tissues and species.[98] Using light and electron microscopy, this group has demonstrated that their process effectively removes nearly all cellular constituents without ultrastructural evidence of damage to fibrous components.

Biochemical tests have confirmed the retention of collagen and elastin and some differential extraction of glycosaminoglycans. Biomechanical testing demonstrated preservation of the matrix structure and mechanics over a physiological loading range.[103] Most interesting, however, was that when the decellularized tissues were inserted into an allogeneic model they showed no inflammation and showed early signs of cellular repopulation.[104] Conversely, when inserted in a xenogeneic model there was evidence of multifocal mixed inflammatory infiltrates and focal destruction of elastin.[97] These latter findings support our previously stated belief that allogeneic interstitial proteins are not immunogenic. Using a similar but less aggressive protocol with Triton X-100, Bader et al demonstrated the capacity to repopulate detergent decellularized valves with xenogeneic endothelial cells in vitro.[99] Unfortunately, these grafts were not implanted and thus the immunogenic potential could not be determined. Other detergents have been reported anecdotally in the literature. A recent case report reported the implantation of a pulmonary valve allograft that had been decellularized with deoxycholic acid and cultured with autologous endothelial cells prior to implantation.[105] At one year follow up the valve is reported to be functioning normally.

Alternatively, various enzymatic treatments to eliminate viable native cells from implant tissues may be used.[100,101] For example, extended exposure to trypsin results in cell death. However, because various proteins including a portion of the type I collagen molecule is sensitive to proteases including trypsin this process will have to be carefully assessed prior to implantation of collagenous matrices into high mechanical stress locations. Bader's group who had previously reported the use detergent-based decellularization has more recently reported a trypsin/EDTA decellularization technique.

In a sheep model they demonstrated almost complete removal of all cells and the effectiveness of in vitro seeding with autologous endothelial and myofibroblasts.[101] Using a sheep model of orthotopic pulmonary valve transplantation they demonstrated reconstitution of endothelial cells, myofibroblasts, and matrix synthesis at three months and thus a viable heart valve. When decellularized valves that had not been repopulated in vitro were implanted into the same sheep model they demonstrated partial degeneration and no interstitial reconstitution at three months, possibly suggesting that the enzymatic decellularization was too harsh to allow for repopulation in vivo. From the same group, Cebotari et al. reported the same decellularization process in human valves with encouraging results.[100] Human implants were not performed.

O'Brien et al have proposed a completely acellular implant created by a proprietary decellularization process.[102,106-109] Unlike the previous techniques which expose the allograft to proteolytic enzymes and detergents which can have potentially destructive effects on the acellular matrix, O'Brien's group uses a combination of aqueous hypotonic solutions which effect the lysis of native cells in the allograft tissue. To prevent the activity of proteases such as collagenase, metal chelators (e.g.: ethylenediaminetetraacetic acid; EDTA) are also added. This approach to allograft processing is a dramatic change in philosophy for this group which, several years prior, were promoting maintenance of cellular viability in their preservation techniques. The results of the acellular valve have been somewhat promising with minimal inflammatory infiltrates, relative maintenance of valve structure, and evidence of interstitial cell in-growth in animal studies. Early results from human trials have demonstrated good hemodynamics. However, there was a lack of endothelialization, sample sizes were small,

and long-term results were not available. Surprisingly, these studies were done in large animals and humans with no evidence that efforts were made to look at the immunology of the valves in larger numbers of animals. A rodent model would be an excellent method to study the basic biology of these tissues.

CRYOPRESERVATION

The beneficial effects of cryopreservation on aortic valve allografts are debatable. When compared to both the older techniques of preservation (chemical, irradiation/ freeze-drying) and the other techniques presently in use (antibiotic and fresh) cryopreservation's superiority is well documented. However, even cryopreserved valves have a finite lifespan. Moreover, recent studies with both animal and human cryopreserved allografts have noted a non-specific immune response (characterized by mononuclear cell infiltration) within weeks of implantation and a predisposition of syngeneic and allogeneic rat aortic valve leaflets to accelerated injury and destruction.[36,39,110]

When Mitchell et al.[63] examined thawed human cryopreserved allografts which were not implanted they found variable autolytic changes, some loss of cellular detail, and a surface devoid of endothelium. Messier et al. compared the effects of cryopreservation in porcine aortic valves using light and electron microscopy, immunohistochemical assays, vital dye exclusion, and silicone rubber substratum assay.[111] When compared to porcine valves which were not cryopreserved, they found the cryopreserved valves had markedly reduced viable cell numbers and damage to both soluble extracellular matrix elements and cell ultrastructure was increased. Kano et al.

assessed the effects of cryopreservation on collagen metabolism in porcine valves.[112] Compared with fresh valves, thawed cryopreserved valves demonstrated maintenance of collagen synthesis, increased collagenolysis, and decreased overall protein synthesis, thus suggesting that cryopreservation results in negative collagen metabolism balance and may have detrimental effects on the valve matrix and its long-term durability.

In order to maintain an adequate inventory of decellularized allografts at an institution the allografts may have to be cryopreserved for later use. Given the evidence which is accumulating for the detrimental effects of cryopreservation on unprocessed allografts it will be essential to investigate the effects of cryopreservation on decellularized matrixes, in particular the immune response, and the potential for cellular in growth. To date there is a paucity of literature in this regard.

SUMMARY

The ideal valve substitute would have the following characteristics: low transvalvular gradient, resistance to infection, low thromboembolic potential, life-long durability, and the capacity to repair, remodel, and possibly even grow. This has been the goal of cardiac surgeons for 40 years. In order to meet these criteria, the valve would have to be a living structure with minimal immunogenicity and intact cellular elements (especially fibroblasts) of either donor or recipient origin. At present, the closest substitute is the cryopreserved allograft which ultimately fails secondary to alloreactive immune response mediated degeneration. We believe that if the immunogenic source could be removed from the grafts, the alloreactive response could be ameliorated and durability improved. This acellular matrix could be capable of repopulation by host cells,

cells that would also be nonimmunogenic and capable of ongoing repair of the valve. Such a tissue could positively impact the outcomes of individuals requiring allograft tissue at the time of cardiac surgery, especially children. The development of decellularized allograft tissue will be the focus of this thesis.

HYPOTHESES AND THESIS AIMS

This thesis focuses on the limitations of cryopreserved allograft tissue used in cardiac surgery with an emphasis on the use of this tissue in congenital cardiac surgery. It aims to further define the humoral immune response to cryopreserved allograft tissue used in congenital cardiac surgery and to find methods to ameliorate the alloreactive immune response to this tissue. **Major hypotheses to be tested include:**

I. **Cryopreserved allograft tissue used in congenital cardiac surgery is associated with donor-specific sensitization.**

Specific aims include:

1. to compare the panel reactive antibodies of infants undergoing congenital cardiac surgery requiring cryopreserved allograft tissue to those undergoing surgery without cryopreserved allograft tissue,
2. to determine the specificity of antibodies generated and compare these results to the HLA type of the donor allograft.

II. **Removal of antigenic cellular elements will ameliorate the cellular and humoral immune response to allograft tissue.**

Specific aims include:

1. to compare current decellularization techniques to identify the most appropriate technique for the study of the immunology of decellularized allograft tissue in a rodent model,

2. to further optimize the decellularization technique identified in specific aim II-1 for use in a rodent model,
3. to define the immunologic response to decellularized allograft tissues in an in vivo rodent model.

III. Decellularization will preserve the biomechanical properties of allograft tissues.

Specific aims include:

1. to determine the biomechanical properties of decellularized allograft tissue using static testing techniques (stress at fracture, elastic modulus, extensibility).

IV. Systemic therapy with intravenous immunoglobulin can prevent sensitization in infants receiving allograft tissue during congenital cardiac surgery.

Specific aims include:

1. to determine whether the immunomodulatory effects of intravenous immunoglobulin can prevent sensitization in infants receiving allograft tissue during congenital cardiac surgery.

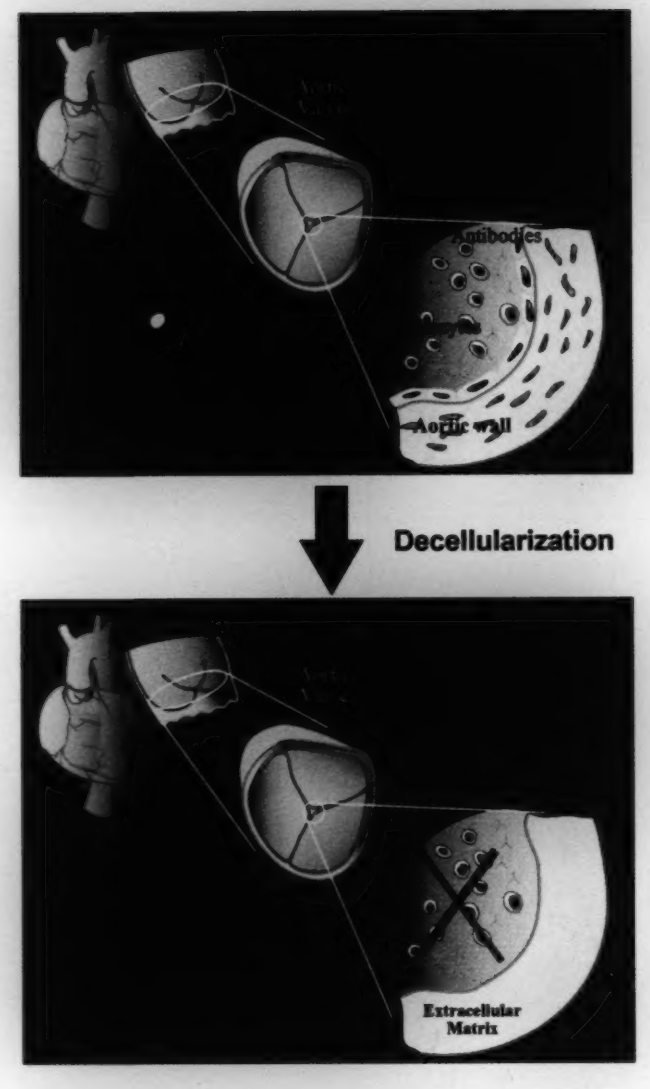

Figure I-1. Summary of rationale for decellularization. It is hypothesized that removal of the cellular elements (endothelial and interstitial cells) in the allograft (i.e. decellularization) will abrogate the alloreactive immune response. This, in turn, will: 1) prevent immune mediated damage to the valve and 2) improve valve durability, and 3) prevent the generation of donor-specific antibodies.

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II

USE OF AN ALLOGRAFT PATCH IN REPAIR OF HYPOPLASTIC LEFT HEART SYNDROME MAY COMPLICATE FUTURE TRANSPLANTATION

INTRODUCTION

The Norwood operation followed by a staged Fontan procedure has become the accepted standard of care for infants born with hypoplastic left heart syndrome (HLHS). While the results of the Norwood operation are steadily improving, the children are left with a single ventricle Fontan circulation which is known to have a reduced life expectancy.[1] It is probable that cardiac transplantation will eventually be required in some, if not most, of these children.

Evidence is accumulating from both clinical[2,3] and laboratory studies[4,5] that cryopreserved allograft tissue used in congenital cardiac surgery appears to be immunogenic in the majority of patients and may elicit an early and intense cellular immune response. Less, however, is known about the humoral response to the cryopreserved allograft tissue. It is possible that the allograft tissue used in the infant's initial repair may sensitize them and jeopardize the success of potential future transplantation. This is particularly concerning given the evidence that the presence of anti-HLA antibodies prior to transplantation have deleterious consequences for the transplant recipient

It has been demonstrated that pretransplant antibodies are associated with early development of high grade cellular rejection and increased annual rejection frequency,[6,7] increased graft vasculopathy,[8] and decreased survival.[9,10] Notably, Jacobs et al.

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recently reported that in pediatric transplantation (median age 130 days), a PRA > 10% was associated with increased 30 day (25%) and longterm (50%) mortality compared with those with a PRA < 10% (8% and 15%, respectively). Moreover, elevated pretransplant panel reactive antibodies (PRA) increase the time on the wait list and complicate perioperative management at the time of transplantation. Thus, the purpose of this study was to assess the anti-HLA antibody response to allograft patches used in the initial repair of HLHS.

MATERIALS AND METHODS

Study Design: A prospective cohort study was conducted to compare the effect of exposure to cryopreserved allograft tissue (patch of allograft adult pulmonary artery) on panel reactive antibody (Figure II-1). PRA levels were assessed preoperatively and at 1, 4, and 12 months postoperatively in two groups of infants undergoing standard congenital cardiac procedures either with or without allograft tissue. The study was approved by the local ethics committee for human research and written consent was obtained from patients (parents).

Study cohort: Twelve infants undergoing aortic arch reconstruction with cryopreserved pulmonary artery patch. There were 10 infants with hypoplastic left heart syndrome undergoing first-stage palliation (Norwood procedure), one infant with transposition of the great arteries with arch hypoplasia/ coarctation (arterial switch and repair of arch with allograft patch), and one infant with tricuspid atresia with arch hypoplasia (repair of interrupted arch with allograft patch). Allograft tissue was provided by comprehensive tissue centres at two Canadian University Hospitals (University of Alberta Hospital,

Edmonton, Alberta; Queen Elizabeth II Health Sciences Centre, Halifax, Nova Scotia).

Control cohort: Ten infants undergoing an arterial switch operation for transposition of the great arteries. No allograft tissue was used for this procedure.

Variables: Preoperative variables to ensure similarity between the two groups included age, gender, preoperative length of hospitalization, and blood product exposure (amount and type). Perioperative factors included duration of crossclamping and cardiopulmonary bypass, use of hypothermic circulatory arrest, and blood product exposure (amount and type). Postoperative variables included length of stay in ICU, length of stay in hospital, and blood product exposure. All infants received CMV negative, leukocyte depleted blood

Donor and Recipient HLA Typing: Donor and recipient Class I and II HLA typing was tested by molecular methodology. Recipient DNA was purified from whole blood using QIAamp® DNA Blood Mini Kit (Quiagen, Valencia, CA). Donor DNA was purified from bone marrow tissue. HLA A, B, and DR antigen typing was performed using the low resolution Micro SSP™ DNA typing kit (One Lambda Inc., Canoga Park, CA). DNA fragments were separated by agarose gel electrophoresis. HLA antigens were determined through a combination of One Lambda DNA/LMT software analysis and manual interpretation of the electrophoresis results.

HLA antibody analysis: Screening for anti-HLA antibodies was performed using the Flow PRA® Screening Test (One Lambda Inc.). This flow cytometric method of simultaneously screening both HLA Class I and Class II PRA utilizes a pool of 30 different Class I and 30 different Class II microbeads coated with purified HLA antigens.[26] The antibodies in the serum that react specifically to the coated HLA

antigens are detected by using a FITC-conjugated antibody against human IgG. Percent PRA can be determined by the percentage of microbeads that react positively to the serum. Serum samples were analysed according to the manufacturer's recommendations. Test control sera included a negative control from One Lambda Inc. (Catalogue number FL-NC) as well as a positive control which was a 1/32 dilution of a local positive pool made from many high PRA patient sera. A 10µL mixture of class I and class II beads as well as control beads were added to every tube. Patient sera (20 µL) were added and the tubes were incubated for 30 minutes. The tubes were washed twice and 100 µL of diluted FITC conjugate (anti human $F(ab')_2$) was added. A final wash step was performed and the beads were analysed using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). Samples that tested positive for the presence of either Class I and/or Class II antibodies were then further tested for specificities using the FlowPRA® Specific Antibody Detection Test kit (One Lambda Inc). Specificity analysis was also performed by the use of single antigen beads if the PRA > 50% (catalogue numbers FL2HD and FL1HD, One Lambda Inc.). In a few cases antibody specificity for class II was also done by ELISA methodology. The ELISA kit used was Class II ID (GTI, Waukesha, WI).

Data analysis/ statistics: All outcomes were expressed as means and standard deviation. Comparisons between continuous data were made with Mann-Whitney U test and comparisons between nominal data were made with Chi-square or Fisher's exact test where appropriate. Differences were considered significant for a value of $P < 0.05$. Simple linear regression analysis was used to assess the relationship between transfusions and PRA. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS, version 11.5).

RESULTS

Patient demographics are summarized in Table II-1. Except for the transposition group being somewhat longer (50.5 ± 2.4 vs 52.6 ± 3.0 cm; P = 0.041), the two groups were well matched preoperatively. Cardiopulmonary bypass time was similar for the two groups (127.1 ± 59.7 vs. 122.6 ± 19.1 minutes; P = 0.314) but the arterial switch procedure for the transposition group required a significantly longer cross clamp time (42.6 ± 27.7 vs. 58.9 ± 11.5 minutes; P = 0.004). The increased use of circulatory arrest for the Norwood procedure is reflected in the significantly increased total circulatory arrest time for the allograft group (28.0 ± 10.6 vs. 2.7 ± 5.2 minutes; P < 0.001). The allograft group also required more blood products perioperatively, especially packed red blood cells (12.3 ± 9.6 vs. 5.3 ± 1.2 units; P < 0.001) and cryoprecipitate (3.8 ± 4.3 vs. 0.9 ± 1.4 units; P = 0.011).

Both groups had minor elevations in PRA preoperatively, most likely reflecting maternally transmitted antibodies (Figures II-2, II-3). By one month postoperatively, there was evidence for a humoral response with modest elevations in PRA for both Class I (19.6 ± 30.1 vs. 3.8 ± 10.6; P = 0.270) and Class II (17.1 ± 27.5 vs. 4.0 ± 5.7; P = 0.792) antibodies. However, at four months there was a significant elevation in PRA for Class I (61.9 ± 39.9 vs. 0; P = 0.002) and Class II (49.3 ± 41.9 vs. 1.8 ± 3.3; P = 0.022) antibodies associated with the use of cryopreserved allograft patches. PRA at 12 months confirmed the persistence of the humoral response for Class I (79.2 ± 21.1 vs. 0; P = 0.008) and Class II (65.6 ± 27.4 vs. 5.2 ± 6.6; P = 0.008) antibodies.

In an attempt to identify the source of sensitization, antibody specificity was analyzed. This confirmed that the many of the antibodies generated were specific for the

HLA type of the donor allograft (Table II-2). Equally important was the failure to generate anti-HLA antibodies in the absence of HLA mismatch in individuals (e.g. patient 7; Table II-2) who were coincidently HLA matched with their donor allograft. In addition, simple linear regression analysis was used to assess the relationship between transfusions and PRA. This suggested a negative relationship between the number of units of packed red blood cells transfused and Class I PRA (β = -2.229; R^2 = 0.286; P = 0.073) and Class II PRA (β = -0.833; R^2 = 0.036; P = 0.554) at 4 months (Figure II-4A). A similar relationship existed between the number of units of platelets transfused and Class I PRA (β = -2.607; R^2 = 0.238; P = 0.108) and Class II PRA (β = -1.658; R^2 = 0.087; P = 0.352) at 4 months (Figure II-4B).

DISCUSSION

Despite previous beliefs that allograft tissue is immunoprivileged, recent investigations have provided evidence that allograft tissues activate alloreactive immune responses. Clinical studies by Baskett et al. correlated enhanced cellular viability of aortic valve allografts with increased antigenicity and increased rate of valve failure.[2] Studies have also correlated HLA mismatch with increased rate of allograft valve failure.[11,12] Other studies in humans and animals have demonstrated an increase in donor-specific cytotoxic[13] and helper T-lymphocyte precursors.[3] Using a rat model our group[4] and others[5] have also demonstrated an early and intense cytotoxic T-lymphocyte response along with complete destruction of valve leaflets in allogeneic rats.

Despite the aforementioned cell-mediated response, there is a relative paucity of definitive evidence for a humoral immune response to cryopreserved allograft tissue in

infants. This is somewhat concerning for infants with HLHS because, despite improved results of the Norwood operation for HLHS.[1] it is probable that many of these children will eventually require cardiac transplantation. The unfavourable impact of elevated PRAs on the outcome of cardiac transplantation is well documented. Elevated pretransplant PRAs significantly increase the risk of early allograft failure and reduced patient survival.[6-10] In a review of 14 535 heart transplants performed between 1987 and 1996 from the United Network of Organ Sharing Registry, it was found that elevated PRA at transplantation significantly increased the relative risk of graft failure ($P = 0.0001$).[9] Moreover, a PRA > 60% was found to be associated with a 2.242 relative risk of graft failure. Such PRA levels were not uncommon in our HLHS population after receiving allograft tissue in the Norwood operation. Jacobs et al. recently reported that in pediatric transplantation (median age 130 days) a PRA > 10% was associated with increased 30-day mortality (25% vs. 8%; $P = 0.178$) and overall mortality (50% vs. 15%; $P = 0.0434$) when compared to children with PRA < 10%.[10] These findings occurred despite aggressive preoperative/ perioperative efforts to reduce the PRA including (in various combinations) intravenous immunoglobulin G (IVIG), cyclophosphamide, mycophenolate mofetil, exchange transfusions, and/ or plasmapheresis. Leech et al. reported evidence of acute or hyperacute rejection on endomyocardial biopsy at postoperative day 7 in three of four highly sensitized patients who received orthotopic heart transplants.[14] Similarly, Itescu et al. reported that pretransplantation anti Class II antibodies were associated with early development of high grade cellular rejection ($P < 0.0001$) and increased annual rejection frequency ($P < 0.001$).[6]

As more sensitive methods for detecting antibodies have become available, the impact of allosensitization has also become even more apparent. Using the more sensitive method of PRA-STAT, Kerman et al. reported that recipients with pretransplant PRA-STAT sera > 10% were at increased risk for graft rejection ($P < 0.05$), more rejection episodes/recipient ($P < 0.02$), and graft rejection within 30 days ($P < 0.001$).[8] The increased sensitivity of the FlowPRA technique was recently reported by Tambur et al.[7] When compared to the CDC (complement-dependent cytotoxicity) method, FlowPRA detected a pretransplant PRA > 10% in 34.8% of the patients who initially tested negative by CDC methodology. Moreover, pretransplant antibodies detected by Flow PRA were highly associated with rejection episodes ($P < 0.001$) and one-year graft survival ($P < 0.004$). Despite the aforementioned negative consequences of an elevated PRA on transplant outcomes, this factor is not an absolute contraindication to surgery. Leech et al. recently reported that in four individuals with elevated PRA and positive prospective lymphocyte crossmatch, good medium-term success (follow up: 17 – 57 months) can be expected with the use of aggressive perioperative immunosuppression despite evidence for acute or hyperacute rejection in three of four patients.[14]

Our study clearly demonstrates an intense humoral response to allograft tissues as evidenced by PRA levels approaching 100% in many of those exposed to such tissue. Moreover, we provided evidence that the PRA level continues to increase with time between 4 and 12 months. Our findings are consistent with those of other institutions.[15-18] Hoekstra et al. found that panel reactive antibodies developed in 78% of 32 recipients of cardiac valve allografts.[16] Smith et al. similarly noted a strong donor HLA-specific antibody response with HLA antibodies detected in 56% of recipients of antibiotic-

preserved allografts and 100% of homovital (fresh) allograft recipients.[17] Hawkins et al. identified a significant increase in alloreactive antibodies in 24 children receiving cryopreserved allografts: at 3.3 months after operation panel reactive antibodies had risen to 92% (1.9% preop) and Class II antibodies had risen to 70%.[18]

In support of the hypothesis that the allograft tissue was responsible for the elevations in PRA, specificity analysis confirmed that a substantial number of antibodies generated were specific for the HLA type of the donor allograft. Specificity analysis did, however, identify antibodies which were not specific for the HLA type of the allograft. One possible explanation for this finding are cross-reactive groups (CREGs).[19] When an individual is mismatched for a HLA antigen they may make antibodies to all the mismatched epitopes on this antigen. These epitopes are shared with other HLA antigens. Most of the antibody reactivity we observed can be explained by cross-reactivity with public epitopes shared by the donor antigen and very few antibody specificities cannot be explained on the basis of CREGs. Moreover, in the case of infant number seven who received an allograft matched for HLA class I antigens there was an absence of a PRA response. This finding, to the best of our knowledge, has not been previously reported in the literature for this population. Others have also reported a failure to develop an elevated PRA in response to allograft tissue; however, in these studies the HLA mismatch was not reported and thus the reason for the failure to develop antibodies is unclear.[12,16]

Admittedly, there is potential for the results to be confounded by the increased use of blood products in recipients of allograft tissue. FFP may contain soluble HLA antigens; however, the exposure to this product did not differ significantly between the two groups. Cryoprecipitate use did differ significantly between the two groups but the exposure to

soluble antigens is usually considered to be negligible with this product. The increased use of packed red blood cells by the HLHS group, however, is concerning. Despite being leukodepleted, there is the chance that some of the children may have developed antibodies in response to these transfusions. In addition, the expression of Class I HLA antigens by platelets could also be a source for sensitization. Despite these concerns, simple linear regression failed to identify a significant relationship between red blood cell or platelet transfusion and PRA levels at four months. In fact, a negative relationship between transfusions and PRA was noted which may suggest an immunomodulatory effect of transfusions.[20] This observation is only speculative and limited by the small sample size in our study. In addition, we have clearly demonstrated that the majority of the antibodies are specific for the HLA type of the allograft, and thus can, cautiously, assume that the impact of blood transfusions is minimal. Furthermore, the difference in PRA levels between the two groups is between 10 fold and infinite whereas there are much smaller differences in the number of blood products used. Lastly, in those instances where the infant undergoing the Norwood procedure coincidentally received an HLA-matched allograft there was no PRA response, again suggesting that it is the immunogenicity of the allograft, not blood product usage, that caused the antibody response.

These findings provide impetus to find methods to reduce the immune response to allograft tissue, to use alternative tissue, or to not use any tissue at all. In a pilot study Shaddy et al. recently demonstrated that a 3-month postoperative course of mycophenolate mofetil significantly, but not completely, abrogated the humoral immune response to valved allografts in children undergoing cardiac surgery. The long term effect

of this therapy on the development of anti-HLA antibodies, however, is still not known and the use of this agent is not without consequences due to its toxicity.[21] Decellularization has the potential to remove immunogenic cellular elements from the allograft tissue. Hawkins et al. reported that decellularized grafts significantly reduce Class I and Class II antibody levels after implantation in children (mean age 8.5 ± 7.9 years).[22] Nine of 14 patients maintained PRA levels <10% in the first year of follow up. These findings are consistent with those in our laboratory investigating the humoral immune response to decellularized allograft tissue in a rat model. HLA mismatch and ABO mismatch has been significantly associated with allograft failure.[11] These findings along with our observation that lack of HLA mismatch is associated with failure to develop antibodies (patient #7) suggests that greater efforts to avoid HLA and ABO mismatch would be beneficial. However, this would require tissue banks much larger than most institutions maintain, a problem that could potentially be overcome by an organ-sharing network.[23] Others have used an autologous[24] or bovine pericardial patch with acceptable results. Lastly, primary repair has been advocated by some groups. Ishino et al. reported that they were able to perform a direct pulmonary artery-to-aorta anastomosis in 85% cases with survival very comparable to that reported by other groups[1] during the same time period, albeit with a 23% recoarctation rate in the survivors.[25]

CONCLUSION

In conclusion, in our experience the use of cryopreserved allograft tissue in the Norwood procedure is associated with a significant humoral response in the majority of patients, especially in those who were mismatched for HLA type. Although the exact

number of hypoplastic left syndrome children treated with surgical palliation who will require subsequent transplantation is unknown, we do know that prior sensitization portends a significant risk for early graft failure and poorer patient survival. Taken together, these findings suggest that the use of allograft tissue in the Norwood procedure may complicate future transplantation. Norwood stage I should preferably be performed without an allograft patch. Alternatives such as an autologous pericardial patch, direct anastomosis, or methods such as decellularization to make the allograft tissue less immunogenic should be considered.

Table II-1. Patient Demographics

Variable[1]	Allograft used	No allograft	P-value
N	12	10	
Age at surgery (days)	8.3 ± 4.8	10.1 ± 8.1	0.821
Gender (M%)	75%	30%	0.084
Length (cm)	50.5 ± 2.4	52.6 ± 3.0	0.041
Weight (kg)	3.4 ± 0.4	3.5 ± 0.7	0.456
XC time (min)	42.6 ± 27.7	58.9 ± 11.5	0.004
CPB time (min)	127.1 ± 59.7	122.6 ± 19.1	0.314
TCA time (min)	28.0 ± 10.6	2.7 ± 5.2	<0.001
PRBC (units)	12.3 ± 9.6	5.3 ± 1.2	<0.001
Platelets (units)	4.8 ± 7.5	1.5 ± 0.7	0.159
FFP (units)	1.3 ± 1.2	0.7 ± 0.8	0.346
Cryo (units)	3.8 ± 4.3	0.9 ± 1.4	0.011

[1]XC time, cross clamp time; CPB time cardiopulmonary bypass time; TCA time, total circulatory arrest time; PRBC, packed red blood cells; FFP, fresh frozen plasma; Cryo, cryoprecipitate

Table II-2. Donor-recipient HLA antigen mismatch and antibody specificities at four months.

Pt	Antigen Mismatches [1,2]		PRA 4 months		Antibody specificities	Donor Specific Antibodies
	Class I	Class II	Class I	Class II		
1.	A2 A11 B51	DR11	0%	0%	None	None
2.	A2 B7 B14 (64 or 65)	DR7 DR15	100%	59%	A2 A11 A23 A24 A25 A26 A29 A30 A31 A32 A33 A34 A68 B7 B13 B27 B44 B45 B49 B55 B57 B60 DR7 DR12 DR15 DR16	A2 B7 DR7 DR15
3.	N/A	N/A	92%	0%	N/A	N/A
4.	A1 A29 B8 B44	DR3 (DR17 or 18) DR7	99%	82%	A1 A11 A29 B8 B27 B44 B45 DR7 DR9 DR17	A1 A29 B8 B44 DR7 DR17
5.	A11 A28 B14 B27	DR3 (DR17 or 18)	17%	0%	None confirmed	None
6.	A24 B50	DR13	60%	93%	A23 A24 B21 (B49 B50) DR13 DR17 DR53 DR103	A24 B50 DR13
7.	None	DR1 DR15	0%	67%	DR1 DR15	DR1 DR15
8.	A2 B14 (64 or 65)	DR13	65%	85%	A2 A11 A23 A24 A25 A26 A30 A68 B57 B65 DR13 DR14 DR17	A2 B14 (65) DR13
9.	A3 B35 B44	DR14	31%	3%	A3 A11 A30 A31 B13 B35	A3 B35
10.	N/A	N/A	98%	88%	A3 A24 A25 A29 A32 A33 B8 B13 B18 B27 B35 B40 B45 B49 B55 B57 B62 B65 DR1 DR9 DR11 DR13 DR15 DR103	A24 B8 B40 DR1 DR13
11.	A11 B27 B61	DR1	81%	15%	A1 A3 A11 A23 A24 A25 A31 A32 B27 B49 B52 B57 DR1	A11 B27 DR1
12.	N/A	N/A	100%	100%	A3 A23 A24 A25 A29 A30 A31 A32 A33 A34 A68 B8 B13 B18 B27 B38 B44 B45 B49 B51 B52 B57 B62 B65 DR7 DR9 DR11 DR12 DR13	No donor typing

[1] Antigen mismatches: donor antigens at which recipient was not matched

[2] N/A: donor or recipient typing was not performed

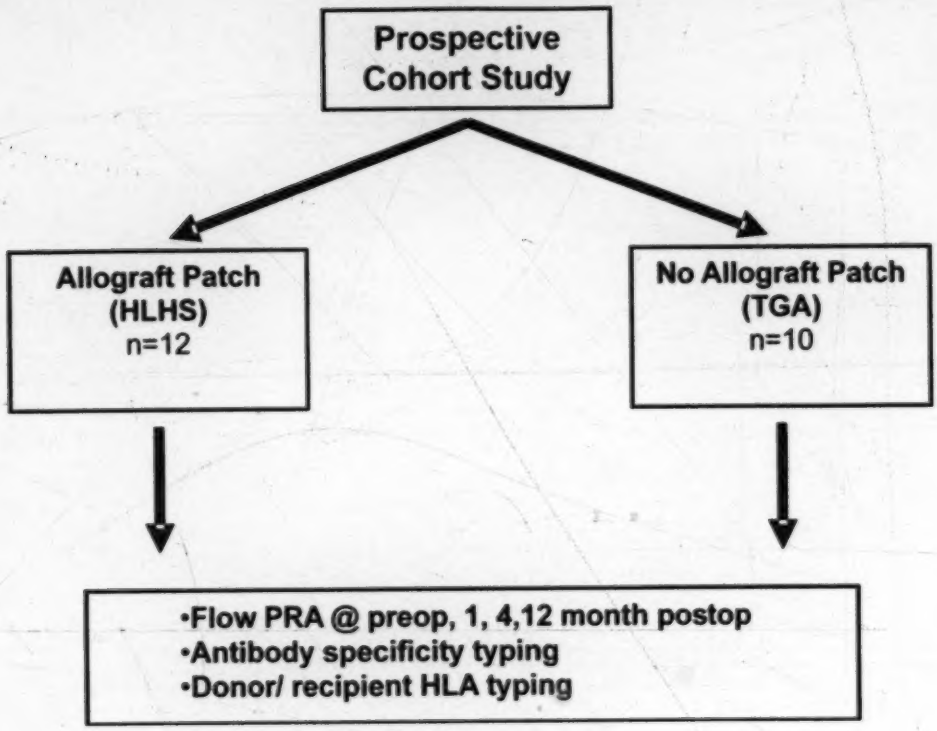

Figure II-1. Study design. A prospective cohort study was conducted to compare the effect of exposure to cryopreserved allograft tissue on panel reactive antibody (PRA) levels at 1, 4, and 12 months postoperatively in two groups of infants (HLHS: hypoplastic left heart syndrome; TGA: transposition of the great arteries) undergoing standard congenital cardiac procedures either with or without allograft tissue.

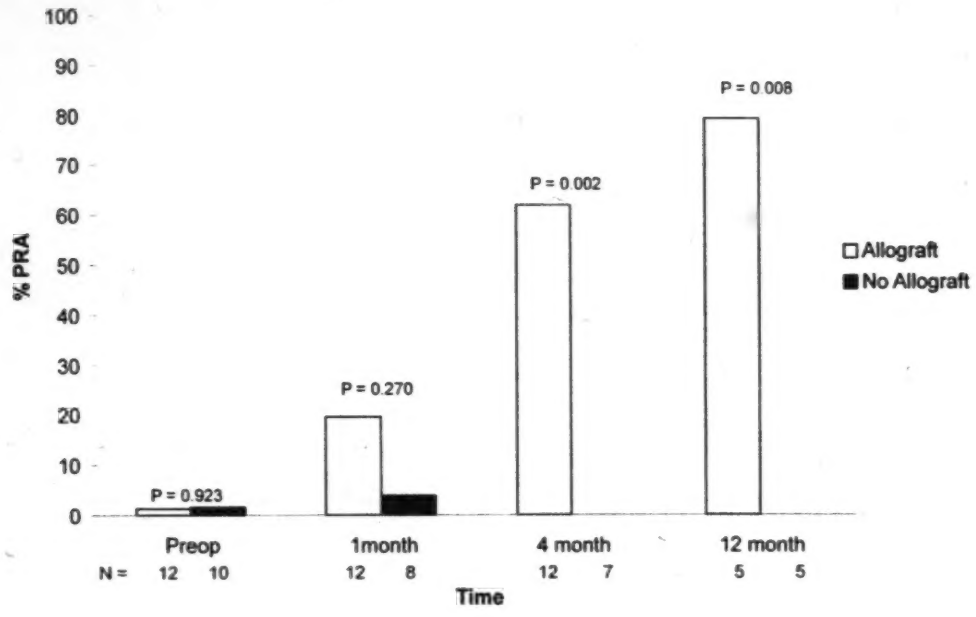

Figure II-2. Class II PRA levels: preoperative and 1, 4, 12 months postoperatively. The use of cryopreserved allograft tissue is associated with the generation of anti-HLA Class I antibodies which becomes statistically significant 4 months after exposure.

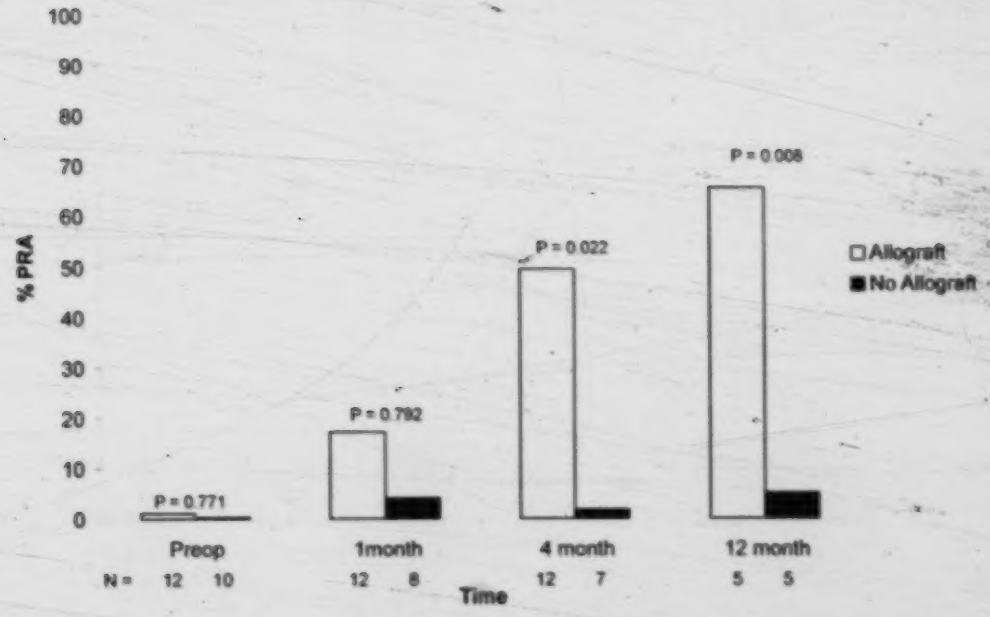

Figure II-3. Class II PRA levels: preoperative and 1, 4, 12 months postoperatively. The use of cryopreserved allograft tissue is associated with the generation of anti-HLA Class II antibodies which becomes statistically significant 4 months after exposure.

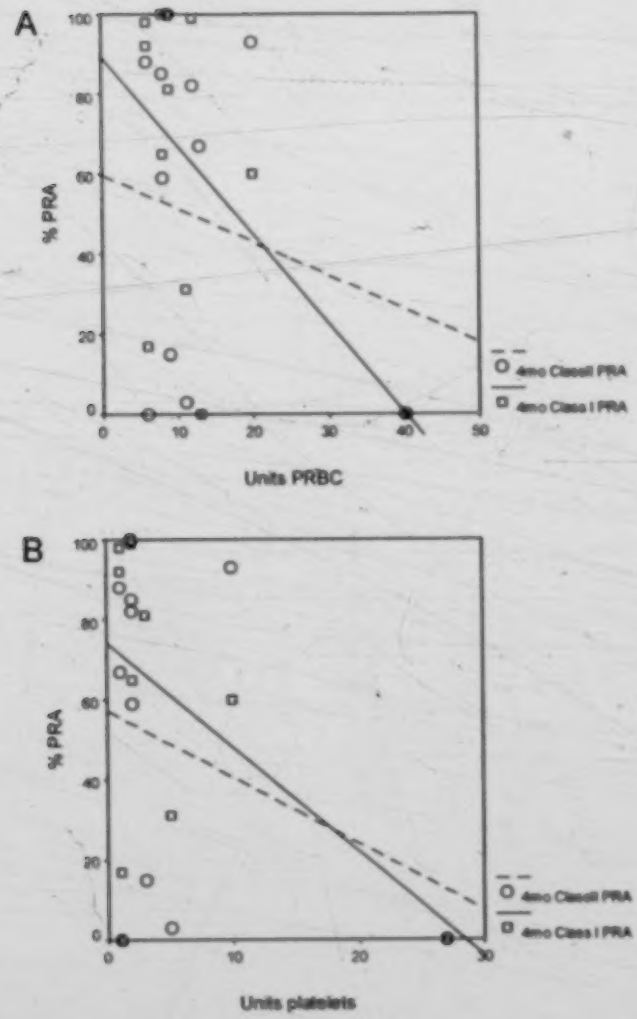

Figure II-4. Relationship between transfusions and PRA. There was a significant negative correlation between packed red blood cells (PRBC) transfused and Class I PRA. (A) Relationship between the number of units PRBC of transfused and Class I PRA (β = -2.229; R^2 = 0.286; P = 0.073) and Class II PRA (β = -0.833; R^2 = 0.036; P = 0.554) at 4 months. (B) Relationship between the number of units of platelets transfused and Class I PRA (β = -2.607; R^2 = 0.238; P = 0.108) and Class II PRA (β = -1.658; R^2 = 0.087; P = 0.352) at 4 months.

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III

COMPARISON OF AORTIC VALVE ALLOGRAFT DECELLULARIZATION TECHNIQUES IN THE RAT

INTRODUCTION

Allograft tissue is used extensively in congenital cardiac surgery and, to a much lesser extent, in adult cardiac surgery. It was previously believed that preservation of cellular viability was essential for long term durability of these tissues and thus great efforts were made to define optimal preservation strategies.[1] However, subsequent clinical research has suggested that this tissue is highly immunogenic and failure of these tissues after implantation is, at least partially, immune-mediated. Prolonged donor ischemia times have been associated with improved allograft durability, suggesting that preserved viability was associated with increased immunogenicity and immune-mediated failure of allograft cardiac valves.[2] Other studies have demonstrated features consistent with immune system activation after implantation of aortic valve allografts including increased number of circulating cytotoxic[3] and helper[4] T cell precursors. Additionally, studies have also demonstrated a donor specific humoral immune response to allograft tissue.[5,6] Despite these indirect findings of allograft rejection, small animal models were required to clearly demonstrate that allograft tissue was associated with profound cell-mediated rejection.[7,8]

Based on findings that the source of allostimulation is the cellular elements[9,10] of the allograft tissue, a number of groups have been investigating decellularization techniques to reduce the immunogenicity of these tissues. To date, the majority of this

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work has been done in large animal models. While this more closely represents the human situation anatomically and allows for detailed biomechanical testing, it limits the opportunity for extensive immunological assays. Small animal models permit the use of larger sample sizes and provide an opportunity to control for immune factors through the use of inbred strains. Rat models are well-described for the assessment of the immunology of aortic valve allografts[7,8,11] and would also provide an excellent model for the study of the immunology of decellularized tissue. A variety of techniques have been described to decellularize allograft tissue in large animal models.[12-14] Before a rodent model can be used to investigate the immunology of decellularization, the effects of decellularization in rodent models needs careful assessment. Thus, the purpose of this study is to compare decellularization techniques in a rat model.

MATERIALS AND METHODS

Experimental Design: Lewis rat aortic valves were decellularized with three different techniques and compared to fresh (nondecellularized) controls. Valves were compared both in vitro and in vivo. In vitro assessment consisted of histologic and immunohistochemical studies to determine valve morphology and cellularity. Hydroxyproline assays were performed to measure collagen content. In vivo assessment of immunogenicity was subsequently performed by implanting decellularized grafts into the infrarenal aorta of allogeneic rats (see Figure V-1, Chapter V) and T cell infiltrates were determined at 1 week postimplantation.

Experimental Animals: Inbred male Lewis (RT1.A^l) and Brown Norway (RT1.A^n) rats were purchased from Charles River (Quebec, Canada) and housed in the institutional

animal care facility with food and water ad libitum for 1 week before experimentation, in accordance with the guidelines of the Canadian Council of Animal Care.[15] Animals were littermates of similar size and age.

Decellularization: Three different decellularization techniques (n = 6/ technique) were assessed in Lewis rat (weight 150g) valves. The first technique (Triton) was detergent-based and was similar to that previously described by Courtman et al.[12] Aortic valves were harvested from donor animals, rinsed with saline solution, and stored in Tris buffer (pH 8.0, 50mM, on ice) for transport (< 4h). The valves were stored in CMRL (90mL, Gibco), FBS (10mL, Sigma) and penicillin-streptomycin solution (penstrep, 0.5mL, Sigma) for 24h at 4°C. Valves were transferred to hypotonic Tris buffer (10mM, pH 8.0) containing phenylmethylsulfonyl fluoride (PMSF, 0.1mM) and ethylenediaminetetra-acetic acid (EDTA, 5mM) for 48h at 4°C. Valves were then placed in a hypertonic Tris buffered solution (50 mM, pH 8.0, PMSF 0.1 mM, EDTA 5mM, KCL 1.5 M) containing 0.5% octylphenoxy polyethoxyethanol (Triton X-100, Sigma) for 48h at 4°C. Valves were then rinsed and placed in Sorensen's buffer (pH 7.3) containing DNase (25mcg/mL, Sigma), RNase (10mcg/mL, Sigma) and $MgCl_2$ (10mM) for 5h at 37°C. Samples were then transferred to Tris buffer (50mM, pH 9.0, Triton X-100 0.5%) for 48h at 4°C. Finally, all samples were washed with phosphate buffered saline (PBS) at 4°C for 72 hours, changing the solution every 24 hours. All stages were performed with constant stirring. Valves were stored in Hank's balance salt solution (HBSS) + penstrep at 4°C until time of implantation.

The second technique (osmotic) was identical to that described above, but without Triton X-100.

The third technique using (trypsin) was similar to that of Cebotari et al.[14] Valves were harvested and transported on ice in HBSS containing penstrep. Valves were washed twice with cold PBS then decellularized with Trypsin 0.5%/ EDTA 0.2 (Sigma) diluted 1:10 with PBS. Decellularization was performed at 37°C for 48 hours in a shaker bath. The trypsin/ EDTA solution was changed twice.

In vivo model: Lewis rat (150g) aortic valve allografts were implanted into recipient Brown Norway (250g) rats using a infrarenal implantation model.[16] Briefly, aortic valves were harvested from heart-beating donors under general anesthesia (Isoflorane) with approximately 5 mm of ascending aorta. Valves were then transplanted into the infrarenal abdominal aorta of the recipient rat using end-to-end anastomoses (10-0 nylon, Sharpoint, Reading, PA) under general anesthesia.

Grafts were implanted as either control (nondecellularized) or decellularized allografts (Brown Norway donor to Lewis recipient; n = 6/ group). Nondecellularized grafts were harvested from donor animals and implanted without delay into recipient animals; decellularized grafts were treated as described above prior to implantation. Tissue was explanted at one week and assessed for T cell infiltrates as described below.

Histology: Tissue was formalin-fixed (10%), paraffin embedded, and serially sectioned (5 μm) for histological and immunohistochemical examination, ensuring valve leaflets were visualized in all sections. Efficacy of decellularization and general morphology was assessed by hematoxylin and eosin (H&E) staining. Extracellular matrix elements (ECM) were visualized with Movat's pentachrome which stains glycosaminoglycans (GAGs) blue, cell nuclei and elastic fibers black, and collagen yellow.

Immunohistochemistry: Immunohistochemistry used standard staining techniques[17] with biotinylated secondary antibodies, a peroxidase avidin/ biotin complex, 3.3' diaminobenzidene as the chromogen, and hematoxylin counterstain. Primary monoclonal antibodies included: anti-PECAM for endothelial cells (sc1506, Santa Cruz Biotechnology, Santa Cruz, CA; 1:250), anti-vimentin for interstitial cells (MCA 862, Serotec, Raleigh, NC; 1:50), and anti-rat MHC which recognizes a monomorphic determinant of rat Class I MHC antigen RT1.A (CL007AP, Cedarlane Labs, Hornby, ON, Canada; 1:50). T cells in explanted valves from the in vivo model were identified with anti-CD3 (sc1127, Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000). Paraffin sections were used for anti-PECAM, anti-vimentin, and anti-CD3 staining and crysostat sections for anti-MHC staining.

Morphometric Analysis: Samples were examined with a light microscope (Nikon, Mississauga, ON, Canada) and images (400x magnification) captured with a digital camera (QImaging, Burnaby, BC, Canada). Morphometric analysis software (Simple PCI, Compix, Cranberry Township, PA) was used to quantify differences between the different decellularization techniques. Anti-vimentin and anti-MHC staining was quantified as percentage of valve leaflet area and valve wall area staining positive for the respective antigen. T cell infiltrates were measured as mean cell counts (CD3+) per leaflet area (number of labelled cells per square mm).[18] Measurements were obtained from 3 representative areas (regions of interest) sampled at the 12, 4, and 8 o'clock positions from two representative tissue cross sections from each animal.

Electron Microscopy: Transmission electron microscopy was performed to assess collagen structure. Samples were prefixed in 2.5% glutaraldehyde in phosphate buffer

(pH 7.2) buffer for a minimum of 1 hour, postfixed in 2% osmium tetroxide followed by brief wash in distilled water, dehydrated in ethanol (50%, 70%, 80%, 90%, 100%), and replaced with propylene oxide. Samples were then embedded in Araldite CY212 mixture/ propylene oxide for 1 hour followed complete Araldite CY212 mixture overnight in a vacuum dessicator. Polymerization was performed at 60°C for 2 days.

Hydroxyproline Assays: Hydroxyproline assays were used to approximate collagen content.[19] Samples were lyophilized and hydrolyzed in 6N HCL. Hydroxyproline was oxidized with chloramine T followed by addition of Ehrlich's reagent. Absorbance was measured at 562 nm and compared to known standards. Values are expressed as μg hydroxyproline/ mg tissue dry weight.

Statistical Analysis: Continuous data is expressed as mean ± standard error of the mean. ANOVA testing was used to compare multiple groups with Scheffe post hoc analysis to compare individual groups. All tests were considered significant with a $P < 0.05$.

RESULTS

Control (nondecellularized) tissue: Normal rat aortic valve leaflets and aortic wall are characterized by a laminar architecture and extensive cellular and stromal elements (Figure III-1A), but leaflets lack the normal trilaminar (fibrosa, spongiosa, ventricularis) structure of human aortic valves leaflets. Immunohistochemisty reveals an intact endothelial layer, extensive interstitial cells throughout the leaflets and the aortic wall, and extensive MHC staining (Figures III-2A-C). The leaflets are predominantly collagen and the aortic wall predominantly elastin (Figure III-2D). This tissue is highly antigenic with 58 ± 2% of the leaflet and 29 ± 2% of the aortic wall staining positive for MHC

antigen (Figure III-3B). Immunogenicity was confirmed by the extensive T cell infiltrates when these tissue were implanted into allogeneic rats for 1 week (3725 ± 357 cell/mm^2 leaflet tissue; Figure III-4). Representative transmission electron micrographs of aortic leaflets (Figure III-5) and aortic wall (Figure III-6) demonstrate highly organized collagen bundles. Hydroxyproline content was 20.7 ± 0.5 μg/ mg dry weight (Figure III-7).

Triton X-100 decellularization: General morphology was characterized by removal of the endothelial cell layer, absence of intact interstitial cells, and preservation of overall ECM structure (Figure III-1B). Absence of staining for endothelial cells is noted in Figure III-2E. Near-complete removal of interstitial cell elements is noted as a significant reduction in staining for vimentin (Figures III-2F,3A). Compared with non decellularized tissue, there was a significant reduction in staining for MHC antigens in both the leaflets (58 ± 2% vs. 1 ± 0%; $P < 0.001$) and aortic wall (29 ± 2% vs. 3 ± 1%; $P < 0.001$) (Figure III-2G, 3B). Reduced immunogenicity was confirmed in vivo with significantly decreased T cell infiltrates (145 ± 35 cell/mm^2; $P < 0.001$ compared to controls) in valve leaflets and a similar reduction in the wall (Figure III-4). Movat's pentachrome staining indicates good overall preservation of collagen and glycosaminoglycans but a slight reduction in the intensity of elastin staining (Figure III-2H). Loss of noncollagen ECM components is suggested by increased hydroxyproline content per dry weight (31.7 ± 1.0 μg/mg; $P < 0.001$) compared to control tissue (Figure III-7). There was no gross evidence of abnormal collagen structure or organization on electron microscopy (Figures III-5,6).

Osmotic lysis decellularization: Similar to decellularization with Triton X-100, osmotic lysis alone was effective in removing endothelial cells and preserving ECM morphology (Figure III-1C). However, this technique was less effective at removing interstitial

cellular elements (Figure III-2J. 3A). Similarly, while this technique significantly reduced MHC staining compared to fresh tissue, it was less much less effective than Triton decellularization at removing MHC antigens in leaflets (Triton vs. osmotic: 1 ± 0% vs. 24 ± 3%; $P < 0.001$) and aortic wall (Triton vs. osmotic: 3 ± 1% vs. 16 ± 1%; $P < 0.001$). This increased staining for MHC antigens correlated with a nonsignificant increase in T cell infiltrates in vivo in the wall of grafts ($P = 0.698$) but not in graft leaflets ($P = 1.0$; Figure III-4) compared to Triton decellularization. Thus, increased antigenicity correlated with only a modest increase in immunogenicity. With osmotic lysis, Movat's pentachrome staining of ECM components was slightly more intense than Triton decellularization (Figure III-2L), and elastin structure in the aortic wall appear slightly less disrupted. Hydroxyproline content per dry weight (25.0 ± 0.6 μg/ mg) was preserved compared to Triton decellularization ($P < 0.001$) and increased compare to control tissue ($P = 0.005$). Collagen structure and organization on electron microscopy appeared normal (Figures III-5,6).

Trypsin decellularization: This technique effected near-complete decellularization but was associated with extensive destruction of the ECM (Figure III-1D). Minimal residual cellularity was confirmed with immunohistochemistry as was the residual antigenicity (Figures III-2M-O). This form of decellularization was associated with extensive destruction of the matrix (Figure III-2P). The tissue had a consistency that made it difficult to implant into the in vivo model. What tissue that was available for examination after implantation demonstrated reduced T cell infiltrates in the leaflets and substantial infiltrates in the wall. Extensive destruction of the ECM and loss of noncollagen elements was suggested by hydroxyproline assays (45.0 ± 1.0 μg/ mg; $P < 0.001$ compared to all

groups). On electron microscopy at 3000x original magnification, the collagen fibres appeared intact but there a loss of organization of the collagen bundles (Figures III-5,6).

DISCUSSION

Despite previous beliefs that allograft tissue used in cardiac surgery was not immunogenic, recent studies have provided evidence that this tissue is extremely immunogenic and is associated with immune-mediated failure.[2,3] These clinical findings have since been supported by animal experimentation.[7,8,20] In addition to cell-mediated immunity, there is accumulating evidence for a humoral immune response to allograft tissue.[5,6] This is particularly relevant in congenital cardiac surgery as there is concern that some children receiving allograft tissue may require future transplantation; for instance, infants undergoing the Norwood procedure for hypoplastic left heart syndrome.[21] It has been well documented in both adult and pediatric heart transplantation that the presence of preformed antibodies negatively impacts short and long-term outcomes.[22,23] Jacobs et al. recently reported that in pediatric transplantation, a PRA > 10% was associated with increased 30 day (25%) and long-term (50%) mortality compared with those with a PRA < 10% (8% and 15%, respectively).[24]

There are two options to attenuate the alloreactive immune response: alter the host (e.g. immunosuppression), or alter the graft. While altering the host has shown benefit in both animal and human trials,[18,25] immunosuppressives are associated with significant toxicity, especially in children.[26] Altering the graft may consist of a bioengineering approach, creating valves from synthetic matrices and then repopulating the graft in vitro or in vivo.[27] Others have investigated decellularization as a method to reduce the

immunogenicity of the allograft tissue.[12,14,28] While much has been published on the biomechanical and biochemical properties of decellularized tissues, there is less documentation of the immunology of these tissues. Small animal models have provided substantial information regarding the immunology of aortic valve allografts and thus would also be essential to study the immunology of decellularized aortic valve allografts. Many decellularization techniques have been described for larger animal models; however, there is a relative paucity of information as to the effect of these techniques on rodent tissues.

We assessed three different decellularization techniques to determine their effectiveness in reducing the immunogenicity of allograft valves. A Triton X-100 based decellularization protocol was associated with a significant reduction in intact cellular elements and anti-MHC staining, suggesting a significant reduction in immunogenicity that was confirmed in vivo. Decellularization with a series of hypotonic and hypertonic buffers was moderately effective at removing cellular intact elements but the tissue still exhibited substantial anti-MHC staining. This gentler technique was associated with somewhat better preservation of the ECM. The failure of osmotic decellularization to reduce antigen staining was most likely due to the inability of an aqueous solution to remove membrane bound MHC antigens after osmotic lysis of cells. Regardless, this protocol reduced T cell infiltrates in vivo to a level approaching that of the Triton protocol. A trypsin protocol previously described for porcine tissues effectively removed all intact cellular elements on H&E staining and significantly reduced antigenicity on anti-MHC staining. Interestingly, however, the trypsin technique was associated with increased immunogenicity (increased T cell infiltrates) compared to other methods. The

reason for this finding is not entirely clear; it may be that tryptic digestion of proteins creates new antigens. In addition to increased immunogencity, the trypsin technique caused substantial disruption of the ECM.

Grauss et al. reported that Triton-based decellularization protocols were ineffective in reducing cellularity of aortic valve allografts.[29] However, this protocol, based on a previous report of Bader et al.,[30] was performed in an isotonic (PBS) solution and only for 24 hours. Our Triton based protocol included both hypotonic and hypertonic solutions which osmotically lysed cells, allowing the Triton X-100 to effectively remove the membrane elements and associated antigens. In addition, we exposed grafts to a total of 96 hours of Triton X-100. Importantly, we were able to use a much lower concentration of Triton X-100 (0.5%) compared to that of Gauss et al. (1%-5%) and still achieve very effective decellularization. This protocol was based on that of Courtman et al. who have published detailed reports on the preserved biomechanical properties of tissues treated with this protocol.[12,31]

The osmotic protocol was similar to the Synergraft protocol described by O'Brien et al.[13] in which cell lysis is initiated in a hypotonic solution (sterile water), followed by equilibration in buffer, treatment with endonucleases, and prolonged washout in isotonic solution. They report that after decellularization that leaflet and conduit cells could not be detected by hematoxylin and eosin staining; this, however, does not imply an absence of residual antigens. In subsequent work, representative sections of decellularized human pulmonary conduit stained with anti-class I and II antisera do identify residual antigens, albeit not approaching the 16% of aortic wall staining which we noted in our osmotically decellularized samples stained for MHC antigens.[28] This residual antigenicity noted may

explain why only 9 of 14 recipients of Synergrafts failed to develop a humoral response (defined as a PRA >10%).[32]

A number of authors have reported trypsin-based protocols. Similar to our findings, Gauss et al. reported extensive destruction of the collagen and elastin elements in the valve. These are important findings as preserving the ECM will be critical for long-term allograft durability. Moreover, preservation of the ECM will be critical for repopulation of the graft by host cells, both in vitro and in vivo. The destructive effects of this protocol are not unexpected: Type I collagen is a major component of heart valves and is also a substrate for trypsin.[33] These trypsin protocols were developed in larger animal models and trypsin has been shown to have a time dependent effect on collagen.[34] More importantly, the small soluble and non fibrillar proteins, proteoglycans, and glycosaminoglycans (GAGs) are readily destroyed by trypsin. This would explain the increase in the proportion of hydroxyproline (collagen) per dry weight. This would also explain the consistency of these tissue: loss of GAGs leads to loss of tissue turgor and thus the poor handling properties of these tissues. Hydroxyproline assays only provide an estimate of the amount of collagen and not the actual integrity of the collagen and thus this assay could not detect whether there was also fragmentation of the collagen. It could be argued that further optimization is required before trypsin is used to decellularize rodent valves.

CONCLUSION

Choosing an optimal decellularization protocol represents a balance between removing cellular elements (thus reducing antigenicity) and preserving matrix integrity.

We chose to focus the majority of our efforts on the antigenicity of the decellularized tissues. Due to the small size of these tissues, biomechanical testing was not possible, but as a surrogate for preserving the extracellular matrix, we quantified the amount of hydroxyproline (collagen) per dry weight of tissue. Decellularization of rat aortic valve allografts with a combination of hypotonic and hypertonic buffers, Triton X-100, endonucleases, and washout in PBS most effectively balances the goals of decellularization: reduction of antigenicity and preservation of ECM. These findings provide critical information for detailed long-term studies of the immunology of decellularized allograft tissue in a rodent model. Such additional studies of longer duration in an in vivo model will be essential to understand the effectiveness of decellularized tissue in preventing cellular and humoral immune responses.

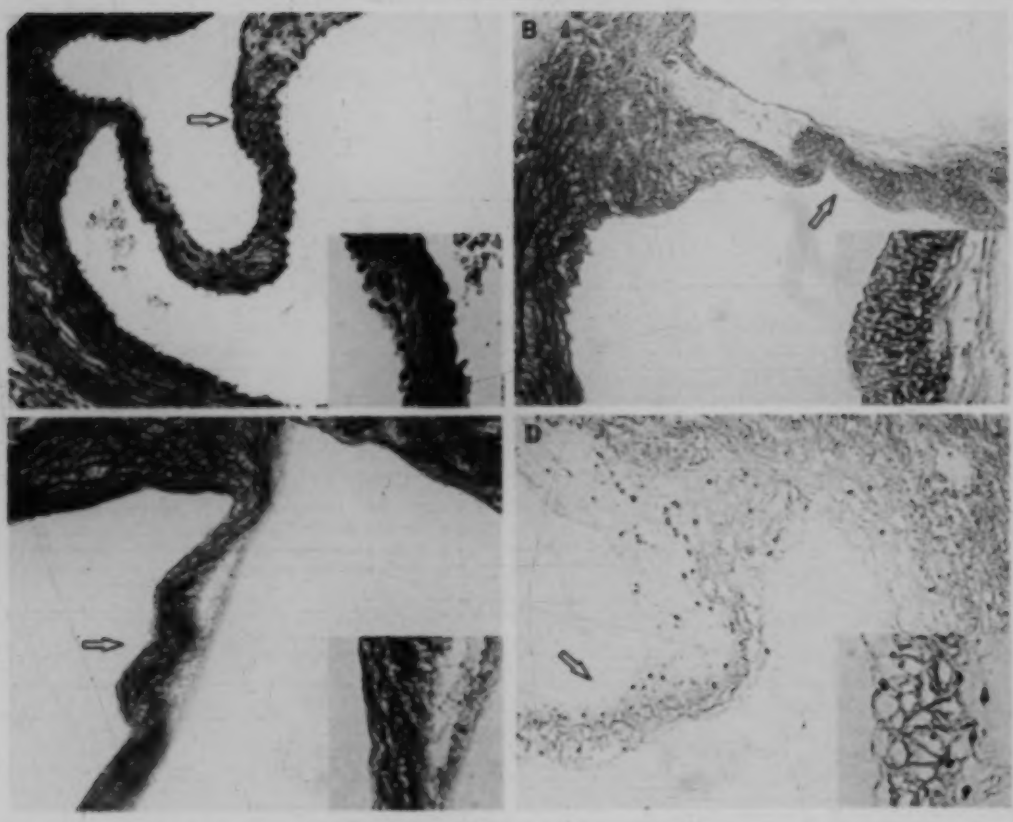

Figure III-1. Hematoxylin and eosin staining of control and decellularized Lewis rat aortic valves. (A) control (non-decellularized), (B) Triton X-100, (C) osmotic lysis, (D) trypsin decellularization. The Triton X-100 protocol was most effective at removing cellular elements and the osmotic protocol was slightly more effective at preserving extracellular matrix staining. Original magnification 200x and 400x (inset). Arrows indicate valve leaflets.

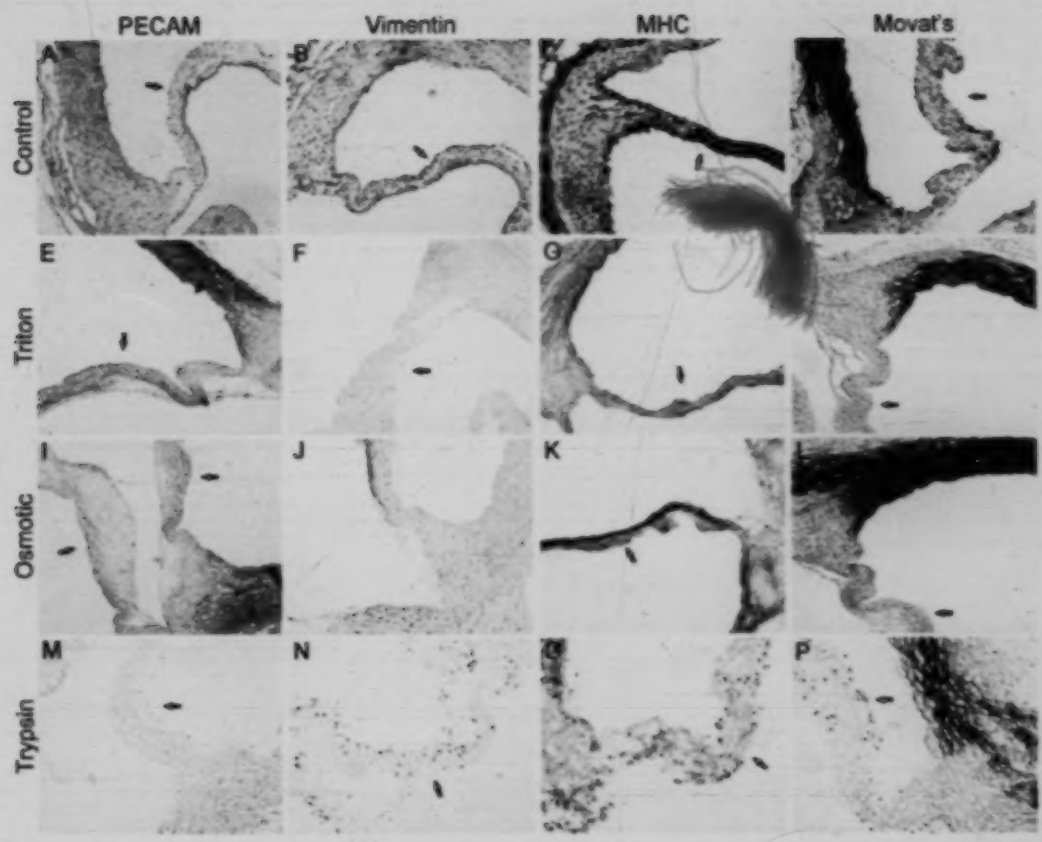

Figure III-2. Comparison of different decellularization techniques using immunohistochemistry and Movat's pentachrome staining. Tissues were stained for endothelial cells (PECAM), interstitial cells (vimentin), major histocompatability complex (MHC), and extracellular matrix (Movat's). Decellularization with the Triton X-100 protocol most effectively reduced cellular elements (PECAM, vimentin) and tissue antigenicity (MHC staining) but was slightly less effective than the osmotic protocol at preserving extracellular matrix (Movat's). Original magnification 200x. Arrows indicate valve leaflets.

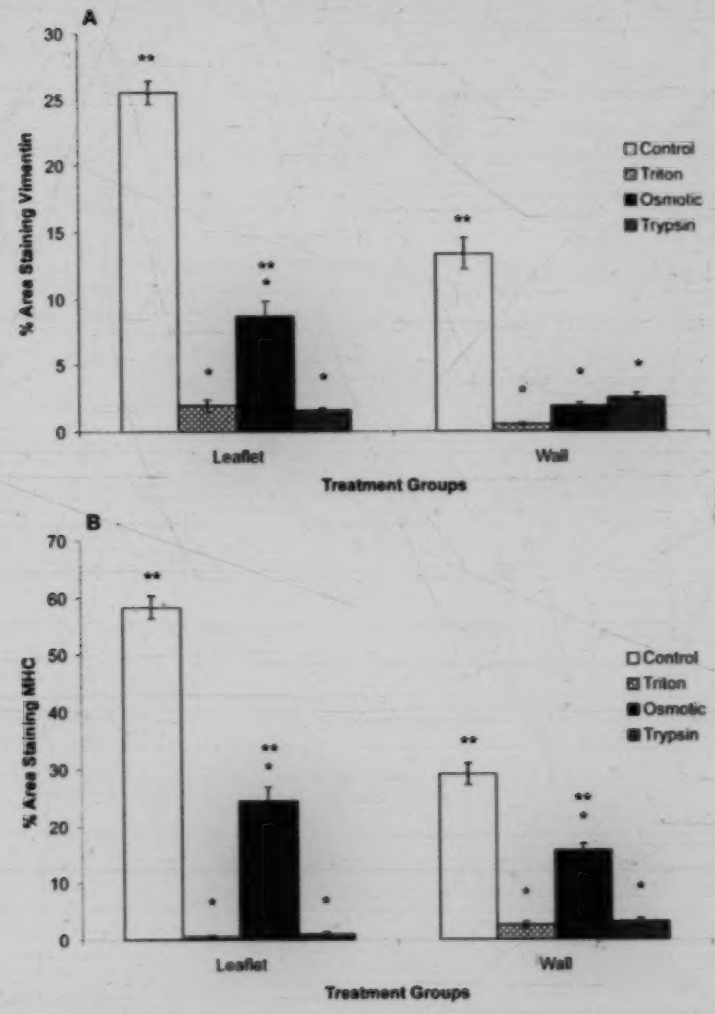

Figure III-3. Summary of morphometric analysis for residual vimentin and MHC staining. Digital images (400x magnification) were captured on a light microscope and morphometric analysis software was used to quantify differences between decellularization techniques. (A) Anti-vimentin and (B) anti-MHC staining was quantified as percentage of valve leaflet area and valve wall area staining positive for the respective antigen. * $P < 0.001$ compared to control; ** $P < 0.001$ compared to Triton. (n = 6/ group).

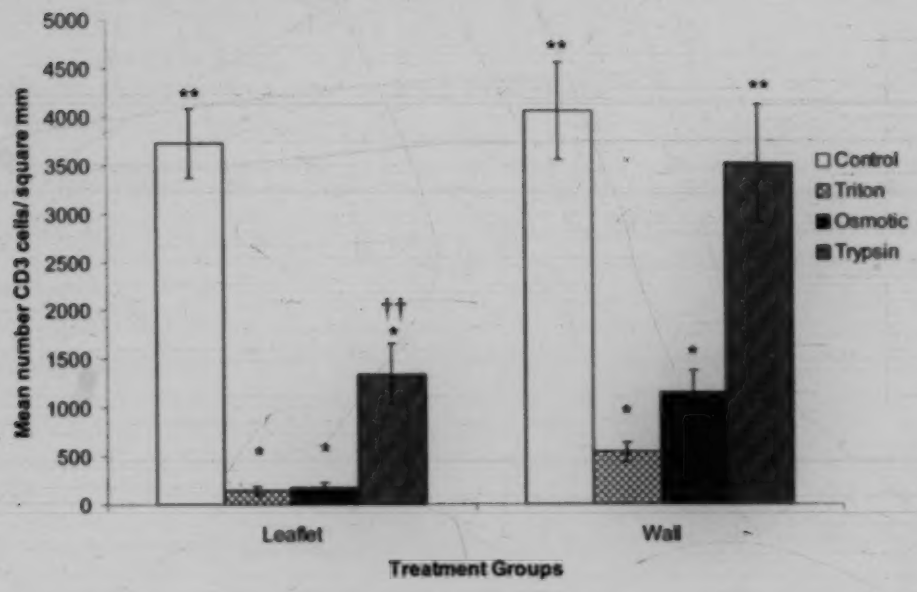

Figure III-4. Summary of morphometric analysis for T cell infiltrates (CD3+) in transplanted allogeneic rats at 1 week. Digital images (400x magnification) were captured on a light microscope and morphometric analysis software was used to quantify differences between decellularization techniques. Results are expressed as the density of T cell infiltrates (number cells per mm^2 of leaflet). * $P < 0.001$ compared to control; ** $P < 0.001$ compared to Triton, †† $P < 0.01$ compared to Triton. (n = 6/ group).

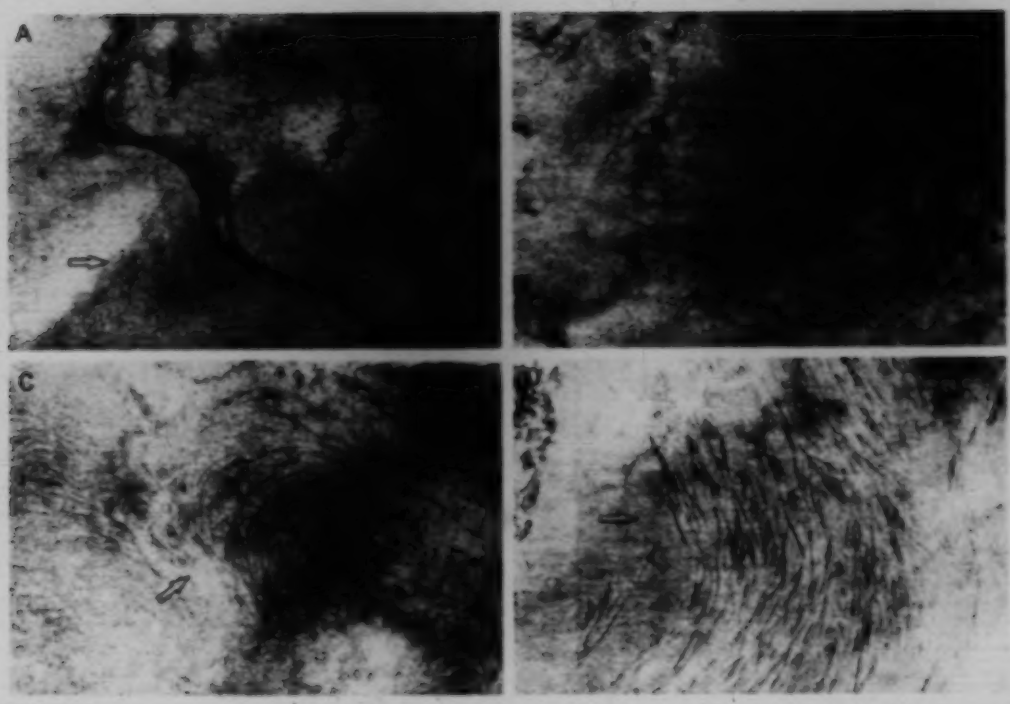

Figure III-5. Representative transmission electron microscopy images of rat aortic valve leaflets. (A) control (non-decellularized), (B) Triton X-100, (C) osmotic lysis, (D) trypsin decellularization. There was no gross evidence of abnormal collagen structure or organization in the Triton X-100 and osmotic lysis groups. Conversely, in the trypsin group the collagen fibres appeared intact but there a loss of organization of the collagen bundles. Original magnification 3000x. Arrows indicate collagen bundles.

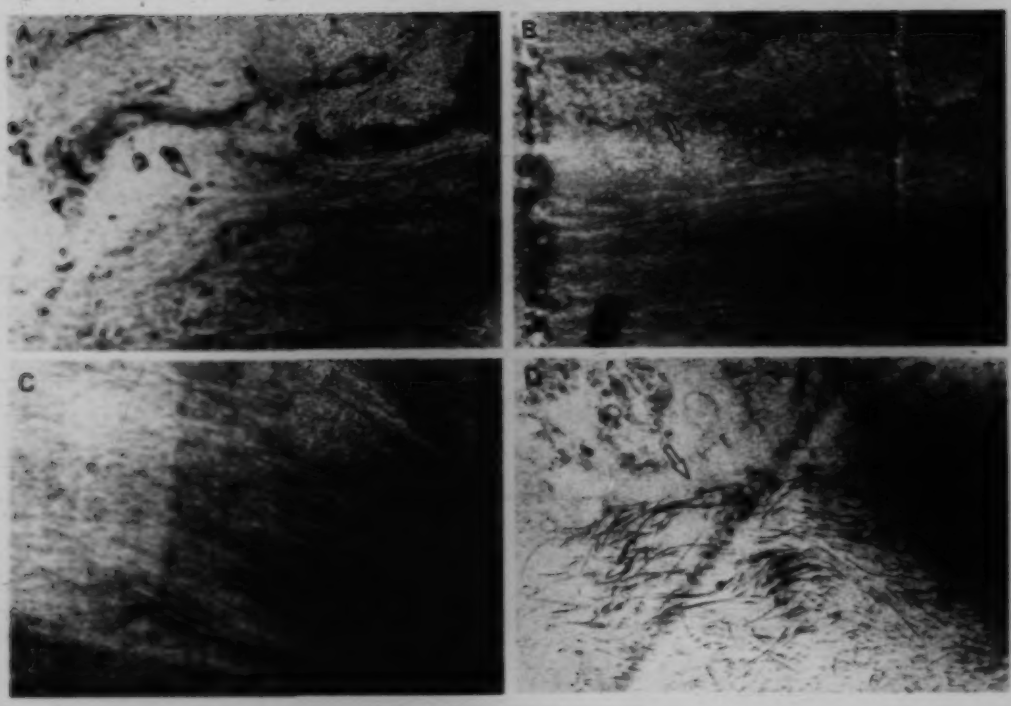

Figure III-6. Representative transmission electron microscopy images of rat aortic wall. (A) control (non-decellularized), (B) Triton X-100, (C) osmotic lysis, (D) trypsin decellularization. Similar to the valve leaflets, there was no gross evidence of abnormal collagen structure or organization in the Triton X-100 and osmotic lysis groups. Conversely, in the trypsin group the collagen fibres appeared intact but there a loss of organization of the collagen bundles. Original magnification 3000x. Arrows indicate collagen bundles.

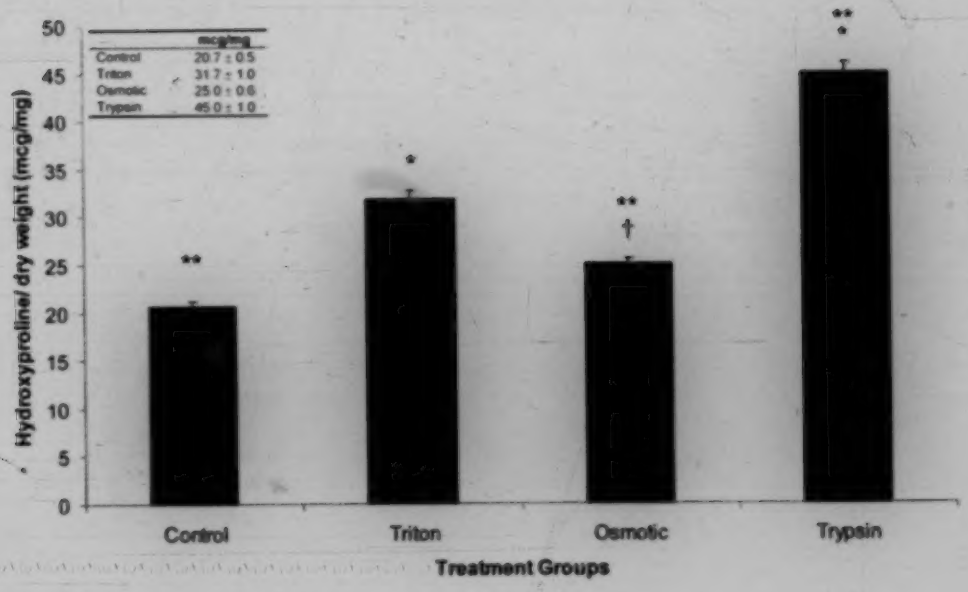

Figure III-7. Results of collagen analysis. Hydroxyproline assays were performed to estimate collagen content. The percentage of collagen per dry weight increased with all three decellularization protocols. These findings are suggestive of removal of soluble extracellular matrix elements (e.g. glycosaminoglycans, small proteins) and preservation of insoluble proteins (e.g. elastin and collagen). * $P < 0.001$ compared to control; † $P < 0.01$ compared to control; ** $P < 0.001$ compared to Triton.

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IV

^{1}H NMR ASSESSMENT OF SAFE TRITON X-100 LEVELS IN DECELLULARIZED RAT AORTIC VALVE TISSUE

INTRODUCTION

Allograft tissue is essential for many of the procedures performed in congenital cardiac surgery. This material is used as patches to enlarge narrowed arteries and as valved conduits to reconstruct diminutive or stenotic structures. Despite previous beliefs that this tissue is immunoprivileged, evidence is accumulating from both clinical[1,2] and animal studies[3,4] that this tissue stimulates a profound alloreactive immune response. This immune response is associated with reduced durability of these tissues. Moreover, our group[5] and others have demonstrated that these tissues stimulate profound (humoral) immunologic sensitization,[6] which may complicate future heart transplantation, if ever required. [7]

One option to reduce the immunogenicity of these tissues is decellularization.[8,9] Most decellularization protocols require the use of detergents. A common detergent used for decellularization process is Triton X-100.[10,11] However, there are concerns that residual Triton X-100 may remain on the decellularized allograft heart valves and Triton X-100 may be toxic when valves are implanted in vivo.[12,13] More specifically, there are concerns that residual Triton X-100 may affect the ability of host cells to repopulate the decellularized matrix. Additionally, there are concerns of systemic toxicity due to leaching of Triton X-100 from the tissue following implantation. Therefore, it is essential to determine whether common decellularization protocols are associated with residual

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Triton X-100, and, more specifically, whether altering final wash times in the decellularization protocol will result in a decellularized allograft heart valve that contains negligible levels of Triton X-100.

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy has been shown to be a rapid, sensitive and effective technique for measuring chemical constituents in biological systems.[14-16] Integration of ^{1}H-NMR spectral signals provides a good quantitative measurement of the relative concentration of species in solution. The amount of Triton X-100 in tissue samples equilibrated with water after the decellularization process for set wash times would therefore be a function of the size and thickness of the heart valve and valve leaflets. By integrating the Triton X-100 peak, it is possible to quantify the amount of Triton X-100 leached into solution and, thus, the relative amount of Triton X-100 left on each rat aortic valve after variable wash times. Using this information the wash time point at which residual Triton X-100 is deemed non-toxic and not a function of wash time can be obtained.

Rodent models have provided invaluable information about the immunology of aortic valve allografts and will be essential for assessing the immunogenicity of decellularized allograft tissue. Before proceeding with such studies it will be essential to determine whether residual Triton X-100 exists in decellularized grafts and to determine optimal washout conditions. Thus, in this study we examined the relationship between phosphate buffered saline (PBS) washout time and Triton X-100 leached from rat aortic valves using ^{1}H-NMR. The ultimate goal of this study was to improve existing protocols for aortic valve decellularization by minimizing possible long-term toxic Triton X-100 exposure.

MATERIALS AND METHODS

Rat Heart Valves: Male Sprague-Dawley rats were obtained from Charles River (Quebec, Canada) and housed in the Surgical Medical Research Institute animal care facility with food and water ad libitum for 1 week before experimentation, in accordance with the guidelines of the Canadian Council of Animal Care[17] and with ethical approval from our institutional Animal Policy and Welfare Committee.

Under general anaesthesia (isoflorane), aortic valves were harvested from donor animals. Tissues were decellularized using methods similar to those described by Courtman et al.[9] Rat aortic valves were rinsed with saline solution and stored in Tris buffer (pH 8.0, 50mM, 4°C) for transport. The valves were then stored in CMRL (90ml, Gibco), fetal bovine serum (FBS, 10 ml, Sigma) and penstrep (0.5ml, Sigma) for 24 h at 4°C. Samples were then placed in hypotonic Tris buffer (pH 8.0/10mM, 250ml) containing phenylmethylsulfonyl fluoride (PMSF, 0.1mM) and ethylenediaminetetraacetic acid (EDTA, 5mM) for 48h at 4°C with constant stirring. The second stage utilized 0.5% octylphenoxy polyethoxyethonal (Triton X-100, VWR) in a Tris buffered solution (PMSF 0.1 mM, EDTA 5mM) for 48h at 4°C with constant stirring. Samples were rinsed with Sorensen's phosphate buffer (pH 7.3), placed in Sorensen's buffer containing DNase (25 mcg/ml, Sigma) and RNase (10 mcg/ml, Sigma) and $MgCl_2$ (10 mM) for 5h at 37°C, followed by Tris buffer (pH 9.0, Triton X-100 0.5%) for 48h at 4°C. Finally, all samples were then washed with phosphate buffered saline (PBS) at 4°C for variable time periods as described below.

Six washout times with PBS were investigated (0h – control, 4h, 6h, 12h, 24h, 48h; n = 6 per time point) in order to determine the shortest and most efficient wash out time to remove residual Triton X-100 from samples.

After each washout time valves were briefly rinsed with water, and each valve was placed in 3 ml of ddH_2O for equilibration. Valves were incubated in the equilibration ddH_2O (4°C) for 2 weeks (14 days) to allow full equilibration of the Triton X-100 within the valve and the external ddH_2O solution (Figure IV-1).

Measurement of Triton X-100: Standard sample spectra were collected on a Varian Unity Inova 500MHz spectrometer (Palo Alto, California). Water (Biopack™, Varian Inc.) pulse sequence was used with a sweep width of 6000Hz, an acquisition time of 4 seconds, and a pre-acquisition delay of one second. More transients were given to samples of lower concentration in order increase signal to noise. Standard sample concentrations ranged from 1.67×10^{-1} M to 1.67×10^{-6} M with corresponding transients ranging from 256 to 18001 (Table IV-1).

Spectra of the unknown samples were acquired using a Varian cryogenic probe. The water pulse sequence was used with a sweep width of 6000Hz, an acquisition time of 3 seconds. The number of transients ranged from 256 to 10656 (Table IV-2) for samples corresponding to the shortest to the longest wash time, respectively. All experiments had a pulse width of 90° pulse width of 7μsec.

The spin lattice relaxation times T_1 of the Triton-X-100 resonances were measured to ensure the differing experimental conditions (relaxation delay and acquisition time) did not compromise the resulting concentration calculations. T_1 denotes the time constant for a resonance to return to equilibrium after being perturbed by a pulse

in an ^{1}H-NMR experiment. Triton X-100 micelles could vary in size with differing concentrations and therefore one would expect to observe different T_1 values for micellular and free Triton X-100 molecules in solution. The T_1 values for the resonances in the methylene region in the micellular Triton X-100 (1.67×10^{-3} M) were to found range from 0.62 – 0.86 seconds with an error of ± 0.016 seconds, whereas the values for monomeric Triton X-100 (1.67×10^{-4} M) were found range from 0.79 – 0.95 seconds with an error of ± 0.006 seconds. Therefore, both the values of acquisition time and pre-acquisition delay used (4 or 5 seconds) were sufficient to allow complete relaxation of the resonances studied between pulses, and the signal intensities can be taken as an accurate reflection of the concentrations of Triton X-100.

All ^{1}H-NMR spectral data were processed using the Advanced Chemistry Development Inc. NMR processing software v6.0 (Toronto, Ontario). The full ^{1}H-NMR spectra of Triton X-100 was first obtained and examined in order to determine the best region to use to quantify the concentration of Triton X-100. It was determined that the methylene region from 3.60 ppm – 3.95 ppm would be a suitable region because it contained intense peaks which were not affected or overlapped by the internal standard DSS peaks. The methylene region was integrated in all spectra and compared with the previously acquired standard curve in order to determine experimental concentration. ***Statistical Analysis:*** Values are expressed as mean ± standard deviation. Statistical significance between time points was calculated using analysis of variance (ANOVA) and Scheffe's post hoc tests for multiple comparisons between individual time points. $P < 0.05$ was considered statistically significant.

RESULTS

Six independent experiments were performed for the rat aortic valves. Figure IV-2 shows a representative 1D ^{1}H-NMR spectra of ddH_2O equilibrated with rat aortic valves which were previously decellularized and washed in PBS for the indicated times.

Figure IV-3 shows the residual concentration of Triton X-100 on each valve as a function of PBS washout time. It can be seen that the concentration of Triton X-100 leached from rat aortic valves decreases with increasing PBS washout time and approaches an asymptote at a washout time of four hours. Thus the residual concentration of Triton X-100 on the rat aortic valves remains relatively constant after four hours.

Table IV-3 summarizes Triton X-100 concentrations from experimental groups in which the isolated rat aortic valves were washed for periods of 0, 4, 6, 12, 24 and 48 hours. At four hours the rat aortic valves show an equilibrating concentration of 7.95×10^{-6} M. ANOVA for all groups resulted in $P < 0.001$, Scheffe post hoc testing shows that the first time point (0 hours) differs from all other time points ($P < 0.001$) and that there were no significant differences between the other time points.

DISCUSSION

Despite forty years experience the search for the ideal cardiac valve replacement continues. The essential characteristics of the ideal valve replacement include durability, absence of thrombogenicity, resistance to infection, the ability to provide normal hemodynamics, and the capacity to remodel, repair, and possibly even repopulate with host cells.[18] Current valve substitutes include bioprosthetic (porcine), mechanical, and transplanted human (allografts; aka homografts). Bioprosthetic valves have limited

durability (10-15 year), mechanical valves require lifelong anticoagulation, and both are prone to infection.[19,20] Allografts, which are essential for many of the complex reconstructions of congenital cardiac disease in children, also have limited durability (10-15 years in adults; less than 5 years in children) after which they require surgical replacement.[21,22] Thus, it can be seen that the ideal valve replacement has yet to be developed.

Clinical studies and work in our lab using animal models have provided strong evidence that allografts stimulate an intense immune response, supporting the concept that immune factors are likely important in the long-term durability of allograft heart valves.[3,4] Allograft heart valves contain living cells, at least initially, and it is these cells which are believed to be the immunogenic stimulus which causes the destruction of these tissues. Moreover, allograft tissue has been demonstrated to stimulate a profound humoral immune response[5,6] that may complicate future heart transplantation if ever required.[7] There are two options to attenuate this alloreactive response: alter the host (e.g. immunosuppression) or alter the valve. Currently available immunosuppression is not acceptable for many valve candidates, especially young children, due to toxicity.[23-25] Altering the valve would require a tissue-engineering approach: we are currently investigating a nonimmunogenic decellularized matrix potentially capable of repopulation by host cells, cells that would also be nonimmunogenic and capable of ongoing repair of the valve.

The detergent Triton X-100 is commonly used in the decellularization of tissue specimens.[10,11] Due to the amphiphilic properties of Triton X-100, it readily disrupts cellular membranes and causes rapid cytolysis. Furthermore, it has been demonstrated

that treatment of cells with Triton X-100 induces typical signs of apoptosis including DNA fragmentation and cleavage of poly(ADP-ribose) polymerase molecules.[12] The toxicity of Triton X-100 could affect the ability of local cells to infiltrate and repopulate the decellularized matrix and may be systemically toxic due to leachate when valves are implanted in vivo.[12,13] Therefore the removal of residual Triton X-100 from tissue that has been treated with this detergent must precede the use of decellularized tissue in vivo. It is imperative that the relative concentration of Triton X-100 that diffuses into solution is less than the concentrations of Triton X-100 necessary to induce cell death in order to have a non-toxic effect in vivo.

^{1}H-NMR has been used extensively to study mixtures of polymer and surfactant aqueous solutions.[14,15] In this study ^{1}H-NMR was used to identify the concentrations of Triton X-100 in ddH_2O solution equilibrated with rat aortic valves at six different wash times. The results from the H_2O solution give an indication as to the amount of Triton X-100 that may leach out of the aortic valves in vivo. As demonstrated in Table IV-3, at a washout time of four hours (corresponding to a Triton X-100 concentration of 7.95×10^{-6} M) the rat aortic valves show marked removal of residual Triton X-100. This concentration of Triton X-100 has previously been shown to be non-toxic.[12,13,26] In addition, we demonstrated that the amount of Triton X-100 as a function of wash time reaches a horizontal asymptote at a wash time of four hours. This implies that at a four hour PBS wash time, the amount of Triton X-100 found on each valve becomes independent of wash time. Therefore wash times greater than four hours for rat aortic valves are unnecessary as they do not significantly change the amount of Triton X-100 leaching out of the valve tissue. Furthermore, previous studies dealing with the removal

of Triton X-100 from other tissues have shown that there is residual irreversibly bound Triton X-100 on sample tissue after wash out or desorption and washout times longer than those suggested above are unlikely to yield additional benefit.[27] Recommended wash times for rat aortic valves should incorporate two factors: the time at which Triton X-100 levels are deemed non-toxic, and the time at which Triton X-100 levels are independent of wash time. Consequently, a four hour wash time is recommended to remove residual Triton X-100 from rat aortic valves.

The results of this study will aid in finding an ideal cardiac valve replacement. Triton X-100 is a critical reagent in the decellularization of allograft tissues and rodent models are essential for studying the immunology of allograft tissues. Demonstrating that the current protocol for removal of Triton X-100 from decellularized valves is effective is critical as it permits additional investigation of the immunologic consequences of decellularization in an established rodent model of aortic valve allografts.[3,4]

This study, however, has raised issues that must be addressed in the future. First of all, the length of time needed to reach an equilibrium point between Triton X-100 and an H_2O solution prior to ^{1}NMR assessment is a variable that can be investigated for optimization. Secondly, the use of ^{1}NMR to determine Triton X-100 concentrations may come under criticism; however, previous studies of a similar nature have confirmed ^{1}H-NMR is an ideal technique for this study.[14-16] Most importantly, the techniques used in this study only assess the amount of Triton X-100 leached out of the tissue. While this provides information on the optimal duration of washout in PBS, it does not directly measure the amount of residual Triton X-100 on the valve tissue. Alternative methods to address this issue could include use of radiolabelled detergent.[28]

CONCLUSION

In summary, ^{1}H-NMR spectroscopy was used to quantify the concentration of residual Triton X-100 leached out of rat aortic valve tissue after decellularization as a function of the decellularization protocol's final wash times with PBS. Based on our findings, we recommend a final wash time of four hours for rat aortic valves. Furthermore, we have shown that the decellularization process is safe and relatively non-toxic in vitro and thus is a viable option for creating an acellular allograft matrix. Ongoing in vivo studies are necessary to determine the immunogenicity and the biomechanical properties of decellularized tissues.

Table IV-1. Correlation between concentration and the number of transients. Standard sample spectra were collected on a Varian Unity Inova 500MHz spectrometer. The water pulse sequence was used with a sweep width of 6000Hz, an acquisition time of four seconds, and a pre-acquisition delay of one second. More transients were given to samples of lower concentration in order increase signal to noise. Standard samples concentrations ranged from 1.67×10^{-1} M to 1.67×10^{-6} M with corresponding transients ranging from 256 to 18001.

Concentration (M)	Number of Transients	Time (min)
1.67×10^{-1}	256	20
1.67×10^{-2}	256	20
1.67×10^{-3}	2048	160
1.67×10^{-4}	2048	160
1.67×10^{-5}	16384	1280
1.67×10^{-6}	18001	1406

Table IV-2. Number of transients at each wash time. Spectra of the unknown samples were acquired using a Varian cryogenic probe. The water pulse sequence was used with a sweep width of 6000Hz, an acquisition time of 3 seconds. All experiments had a pulse width of 90° pulse width of 7μsec.

Wash Time (hours)	Number of Transients	Experiment Time (min)
0	256	20
4	256	20
6	512	40
12	1024	80
24	8096	630
48	10656	832

Table IV-3. Measurement of Triton X-100 concentrations with PBS washout time.

PBS Washout time (h)	Concentration of Triton X-100 (x 10^{-6} M)[a, b]
0	137 ± 31
4	7.95 ± 3.66
6	2.76 ± 1.36
12	2.67 ± 0.46
24	10.6 ± 7.30
48	1.83 ± 0.43

Values are mean ± SD (n = 6).

[a] Triton X-100 concentrations from experimental groups in which the isolated rat aortic valves were washed for periods of 0, 4, 6, 12, 24 and 48 hours.

[b] increased concentration at 24 hours consistent with critical micellar concentration for Triton X-100.

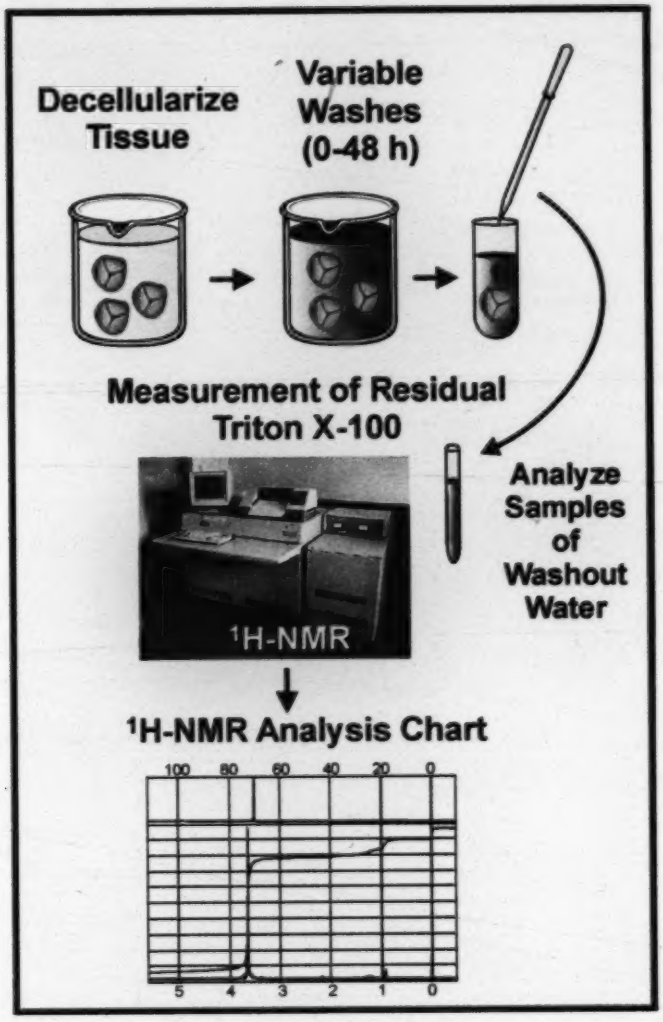
Decellularize Tissue
Variable Washes (0-48 h)
Measurement of Residual Triton X-100
Analyze Samples of Washout Water
¹H-NMR
¹H-NMR Analysis Chart
100 80 60 40 20 0
5 4 3 2 1 0

Figure IV-1. Study methods. Valves were decellularized then washed for variable times periods (0, 4, 6, 12, 24 and 48 hours) in phosphate buffered saline. After each washout time, valves were placed in 3 ml of ddH_2O for equilibration. Valves were incubated in the ddH_2O (4°C) for 2 weeks to allow full equilibration of the Triton X-100 within the valve with the external ddH_2O solution . Sample spectra were collected on a Varian Unity Inova 500MHz spectrometer to determine residual Triton X-100 levels.

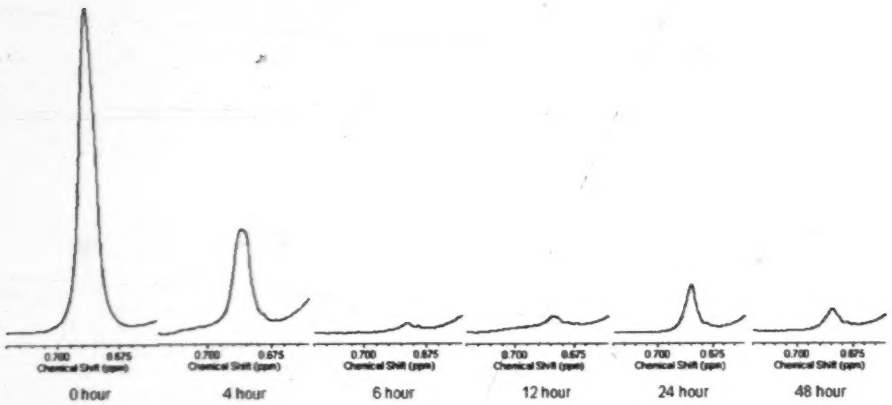

Figure IV-2. Sample 1D 1H-NMR spectra of H2O equilibrated with rat aortic valves at different wash times. There was a substantial reduction in ^{1}H-NMR signals noted after four hours of washout.

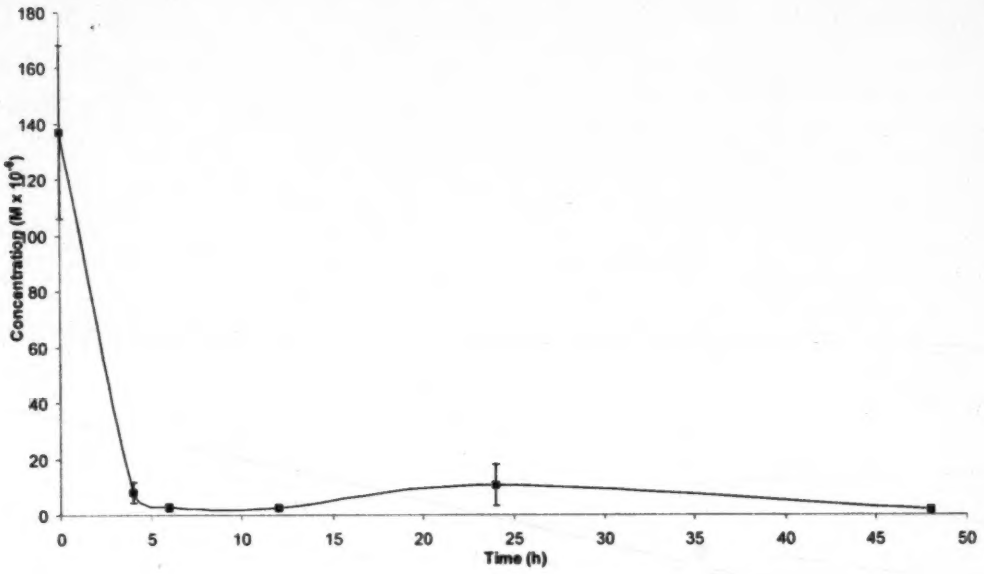

Figure IV-3. Plot of residual Triton X-100 concentrations vs. washout time. Each time point represents the mean ± SD for 6 independent observations. There was a significant reduction in ^{1}H-NMR signals by four hours which decreased minimally with longer washout periods. Increased concentration at 24 hours consistent with critical micellar concentration for Triton X-100. (n = 6/ time point).

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V

DECELLULARIZATION REDUCES THE IMMUNE RESPONSE TO AORTIC VALVE ALLOGRAFTS IN THE RAT

INTRODUCTION

Allograft tissue is used extensively in congenital cardiac surgery as valved conduits and patches to replace or reconstruct diminuitive and malformed structures. While the cryopreserved allograft valve has acceptable results in adults with freedom from reoperation approaching 69% at 15 years[1] failure is much more rapid in children. Freedom from reoperation has been reported as low as 69% at 48 months in children under 2 years of age.[2] It was previously believed that this tissue was immunoprivileged. However, increasing evidence from laboratory[3,4] and clinical investigations[5,6] has clearly demonstrated that this tissue is immunogenic and stimulates an intense cell-mediated immune response. This immune response has been implicated in the failure of these tissues. Equally important is the more recent observation by our group[7] and others[8] that these tissues also stimulate intense humoral responses with panel reactive antibodies (PRA) approaching 100%. While the evidence linking this humoral response to allograft failure is less clear, there is concern that these preformed antibodies may complicate future cardiac transplantation, should it be required.[9] An elevated PRA has been associated with early development of high grade cellular rejection and increased annual rejection frequency,[10,11] increased graft vasculopathy,[12] and decreased survival.[9,13,14]

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It was previously believed that preserving cellular viability was essential for long term valve durability.[15] Consequently, methods had been developed to prepare allograft tissue with the intention of maintaining cellular viability. However, more recent investigations have correlated enhanced allograft viability with increased immunogenicity and increased rates of failure.[16] There is increasing evidence that the source of the alloreactive immune response is the cellular elements of the allograft.[17] These findings, in part, have prompted the recent interest in tissue-engineering of valves, either creating valves from synthetic matrices or through decellularization techniques to remove the immunogenic cellular elements of the allograft tissue. It is hypothesized that creation of an acellular matrix will abrogate the alloreactive immune response, improving valve durability and possibly preventing antibody formation. The purpose of this study was to investigate the cellular and humoral immune response to decellularized allograft tissue in a rat allograft model.

MATERIALS AND METHODS

Experimental Animals: Inbred male Brown Norway (RT1.A^n) and Lewis (RT1.A^l) rats (weight 175 to 250 g) were purchased from Charles River (Quebec) and housed in the institutional animal care facility with food and water ad libitum for 1 week before experimentation, in accordance with the guidelines of the Canadian Council of Animal Care.[18]

Surgical Technique: Aortic valve grafts were implanted into recipient animals according to a well-established infrarenal implantation model (Figure V-1).[3,19] Briefly, this technique involves removing the aortic valve with approximately 5 mm of ascending

aorta from a donor rat and transplanting it into the infrarenal abdominal aorta of the recipient rat using end-to-end anastomoses (10-0 nylon, Sharpoint, Reading, PA) under general anaesthesia (Isoflorane).

Grafts were implanted as either nondecellularized (fresh) or decellularized grafts in both syngeneic (Lewis donor to Lewis recipient) or allogeneic (Brown Norway donor to Lewis recipient) combinations (n = 6/ time/ group). Nondecellularized grafts were harvested from donor animals and implanted without delay (<30 minutes) into recipient animals; decellularized grafts were treated as described below prior to implantation.

Decellularization: Tissues were decellularized using methods similar to that previously described by Courtman et al[20]. Aortic valves were harvested from donor animals, rinsed with saline solution, and stored in Tris buffer (pH 8.0, 50mM, on ice) for transport. The valves were stored in CMRL (90mL, Gibco), FBS (10mL, Sigma) and penicillin-streptomycin (penstrep; 0.5mL) for 24h at 4°C. Samples were placed in hypotonic Tris buffer (10mM/ pH 8.0) containing phenylmethylsulfonyl fluoride (PMSF, 0.1mM) and EDTA (5mM) for 48h at 4°C. Next, samples were placed in 0.5% octylphenoxy polyethoxyethonal (Triton X-100, Sigma) in a hypertonic Tris buffered solution (50 mM/ pH 8.0; PMSF 0.1 mM, EDTA 5mM, KCL 1.5 M) for 48h at 4°C. Samples were then rinsed with Sorensen's phosphate buffer (pH 7.3) and placed in Sorensen's buffer containing DNase (25mcg/mL), RNase (10mcg/mL) and $MgCl_2$ (10mM) for 5h at 37°C. Samples were then transferred to Tris buffer (50mM/ pH 9.0, Triton X-100 0.5%) for 48h at 4°C. Finally, all samples were washed with PBS at 4°C for 72 hours, changing the solution every 24 hours. All stages were performed with constant stirring. Valves were stored in HBSS + penstrep at 4°C until time of implantation.

Efficacy of decellularization was confirmed with staining for overall morphology (hematoxylin and eosin), endothelial cells (anti-CD31; sc1506, Santa Cruz Biotechnology, Santa Cruz, CA), and interstitial cells (anti-vimentin; MCA 862, Serotec, Raleigh, NC) using methods described below.

Immunohistochemistry: Tissue was harvested for histology at 1, 2, and 4 weeks. Samples were formalin-fixed (10%), paraffin embedded, and serially sectioned (5 μm) in a transverse plane for histological and immunohistochemical examination, ensuring valve leaflets were visualized in all sections. Immunohistochemistry involved standard staining techniques with biotinylated secondary antibodies, a peroxidase avidin/ biotin complex, and 3.3' diaminobenzidene as the chromogen. Primary monoclonal antibodies for T cells (anti-CD3; sc1127, Santa Cruz Biotechnology, Santa Cruz, CA) and cytotoxic T cells (anti-CD8; sc7970, Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Tissue Analysis: Samples were examined with a light microscope and images captured with a digital camera (QImaging, Burnaby, Canada). Morphometric analysis was performed to measure cell counts per area (Simple PCI, Compix, Cranberry Township, PA). Leaflet cell density (number of labeled cells per square mm) was obtained from sampling of the entire leaflet area from representative tissue cross sections from each animal. Cell counts were obtained from the average cell density of 3 representative areas sampled at the 12, 4, and 8 o'clock positions from two representative tissue cross sections from each animal.

Flow Cytometric Crossmatch:

SERUM HARVEST: At the time of sacrifice at 2, 4, and 16 weeks, rats were exsanguinated into 10 mL phlebotomy tubes (n = 6/ group/ time point). Blood was allowed to clot and

the tubes were centrifuged for 5 minutes at 1500 rpm. Serum was transferred to 5 mL polypropylene tubes and stored at -86°C until needed.

CELL ISOLATION: Spleens were harvested from Brown Norway rats and mechanically dissociated between sterile slides. Splenocytes were isolated through repeated washing with saline, centrifugation (1500rpm x 5min), and red blood cell lysis (RBC lysis buffer: 0.15M NH_4Cl, 1.0mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.3). Cells were resuspended in DMSO and FBS and frozen at -86°C until needed.

CELL PREPARATION: Cells were thawed and reconstituted in saline. Cell number was counted in a hemocytometer with Trypan blue. 10^6 cells were washed with saline, centrifuged (1500rpm x 5 min), combined with 100 µL of serum from recipient rats (1:50 dilution; in FACS buffer [1%FBS in PBS]), and incubated at 37°C for 30 minutes. Cells were washed twice with FACS buffer, centrifuged, and incubated with 100 µL secondary antibody (FITC-conjugated $F(ab')_2$ fragment goat anti-rat IgG, Fc_γ fragment specific; Jackson ImmunoResearch Laboratories Inc, 112-096-008, 1:200 dilution in FACS buffer) at 4°C for 30 minutes in the dark. Cells were washed with FACS buffer, centrifuged, resuspended in FACS buffer, and analyzed by flow cytometry (BD FacsCalibur, San Jose, CA) for mean channel fluorescence intensity which represents antibody binding to target cells. A right-ward shift indicated the presence of serum antibodies.

Statistical Analysis: Continuous data is expressed as mean ± standard error of the mean. ANOVA testing was used to compare multiple groups with Scheffe post hoc analysis to compare individual groups. All tests were considered significant with a $P < 0.05$.

RESULTS

Samples of decellularized tissues are shown in Figure V-2. These images confirm the efficacy of the decellularization process in removing the majority of cellular elements. In the fresh (nondecellularized) tissue there was extensive cellularity, an intact endothelial layer, and a laminar architecture. In the decellularized tissue there was an absence of intact cells, complete removal of the endothelial layer, and relative preservation of the extracellular matrix. Some residual cellular fragments were detected by vimentin staining.

Representative sections of tissue explanted at 1 week are illustrated in Figure V-3. Most notable in these images is the intense T cell infiltrates and leaflet thickening in the allogeneic nondecellularized tissues as compared to the decellularized tissues. This difference in T cell infiltrates was quantified by morphometric analysis and summarized in Figure V-4A . Scheffe post hoc analysis confirmed a significant decrease in CD3+ T cell infiltrates for allogeneic decellularized tissue as compared to allogeneic non-decellularized tissue at 1 (79 ± 29 vs. 3310 ± 223; $P < 0.001$), 2 (26 ± 11 vs. 109 ± 20; $P = 0.004$), and 4 weeks (283 ± 122 vs. 984 ± 145; $P < 0.001$). There was a modest elevation in CD3+ T cell infiltrates at 1 week in the syngeneic non decellularized group which differed slightly from allogeneic decellularized tissues (703 ± 140 vs. 79 ± 29, respectively; $P = 0.013$). Otherwise, the T-cell infiltrates of the decellularized tissue were similar to that of the control non-decellularized and decellularized syngeneic grafts. Decellularized grafts, however, were very thrombogenic as evidenced by thrombosis of the sinus of Valsalva (Figure V-3D). At 16 weeks the grafts were completely thrombosed with complete loss of valve leaflets, precluding meaningful analysis. Staining for cytotoxic CD8+ T cells (Figure V-4B) confirmed that the majority of the infiltrates were

cytotoxic T cells. There was a similar decrease in CD8+ T cell infiltrates in the decellularized allograft tissue at all time points.

Flow cytometric crossmatch identified a significant reduction in antibody production to levels that were similar to those of syngeneic nondecellularized grafts. Representative histograms at 16 weeks are provided in Figure V-5. The allogeneic decellularized grafts were characterized by a left-ward shift of the histogram compared to allogeneic non decellularized grafts, indicating the failure to generate antibodies to decellularized allogeneic grafts. Similar findings were noted at 2 and 4 weeks, the former being less pronounced. Flow cytometry mean channel fluorescence intensity was used to quantify these results and values are summarized in Figure V-6. Scheffe post hoc analysis confirmed the difference between decellularized allografts and non-decellularized allografts at 2 (19 ± 1 vs. 27 ± 3; $P = 0.033$), 4 (35 ± 2 vs. 133 ± 29; $P = 0.001$), and 16 weeks (28 ± 2 vs. 166 ± 54; $P = 0.017$).

DISCUSSION

Allograft tissue is used extensively in cardiac surgery. In adults, allograft valves are used for reconstruction of the right ventricular outflow tract in the pulmonary autograft (Ross) procedure and they provide an important option for reconstruction of the aortic root, especially in cases of endocarditis. Allograft tissue plays an even more important role in congenital cardiac surgery where it is used both as valved conduits and as patches to reconstruct congenital abnormalities. Despite the advantages of absence of thrombogenicity, resistance to infection, and excellent hemodynamics, allograft valves fail with a predictable time course which is particularly rapid in young children.[1,2]

Allograft tissues contain living cells, at least initially.[5] Thus, it is not surprising that studies have demonstrated that these tissues stimulate a profound cell-mediated immune response with diffuse T cell infiltrates within one week of implantation in an animal model.[3,4] The progressive failure of the allograft valve has been attributed to this alloreactive immune response. More recently, evidence has been accumulating that allograft tissue used in congenital cardiac surgery also stimulates a profound humoral response.[7,8] The role of this humoral response in allograft failure is less clear. However, more concerning is the impact that the presence of preformed anti-HLA antibodies will have on future cardiac transplantation,[9] a possibility in many children with congenital heart disease undergoing palliative procedures such as the Norwood operation for hypoplastic left heart syndrome.

There are two options to attenuate this alloreactive response: alter the host (eg. immunosuppression) or alter the valve. Although shown to be beneficial in animal models, currently available immunosuppression is not acceptable for many valve candidates, especially young children, due to toxicity. Altering the valve to reduce its immunogenicity is another approach. As previously mentioned, it is believed that the cellular elements are the antigenic stimulus for the alloreactive immune response and thus decellularization has been proposed to reduce the antigenicity of these tissues. Numerous protocols have been proposed to decellularize tissues.[20-23] A similar number of studies have been performed to assess the effectiveness in removing cellular elements and to assess the biomechanical properties of decellularized tisuues. However, there is a relative paucity of detailed studies investigating the immunologic consequences of decellularization of these tissues. Thus the current study used a modification of a

previously described decellularization technique[20] and a well-established allograft heart valve implantation model[3,19] to investigate the cellular and humoral immune response to decellularized allograft tissues.

In the present study we clearly demonstrated that decellularization of aortic valve allografts reduces the aforementioned cellular immune response. Similar to previous reports allogeneic nondecellularized grafts were associated with significant CD3+ and CD8+ T cell infiltrates in aortic valve leaflets by one week following transplantation, rapidly decreasing in the following weeks.[3] Decellularized grafts, on the other hand, were associated with significant reductions in T cell infiltrates which were similar in density to those of syngeneic controls. The explanation for the anomalous increase in CD3+ T cell infiltrates in the nondecellularized grafts at 4 weeks is not entirely clear. This finding was not noted in the CD8+ T cells counts. Thus, it leads us to believe that the increased CD3+ T cell counts at 4 weeks may represent infiltration of CD3+ cells other than CD8+ T cells, perhaps CD4+ T cells. These findings are consistent with previous work by our group and others. Legare et al. demonstrated a nadir of infiltrates at 2 weeks in allogeneic aortic valve transplants.[3] Similarly, Green et al demonstrated a nadir of infiltrates in the intima and media of aortic valve allografts at 14 days which rebounded somewhat at 28 days.[4] Moreover, Green et al demonstrated an increased proportion of CD4+ T cells and a decreased proportion of CD8+ T cells at 28 days compared with 7 and 14 days.

Unfortunately, the decellularization exposed the extracellular matrix and thus the graft was highly thrombogenic. The model used was a non-working model with the valve leaflets remaining open throughout the cardiac cycle. In contrast to a working model,

there is no "backflow" in the sinuses of valsalva during diastole leading to stasis and thrombosis. Whether this thrombosis would occur in a working model is unknown. The impact of this thrombosis on cellular infiltrates is not entirely clear. However, because the allogeneic decellularized grafts had a paucity of T cell infiltrates similar to syngeneic nondecellularized controls, it could be cautiously assumed that the impact is minimal. Moreover, the humoral response to the allografts closely mimics the cellular data presented.

Flow cytometry demonstrated that decellularization similarly reduces the humoral response to allograft tissue. Decellularization was associated with a profound reduction of antibodies to the level of syngeneic tissues. These animal studies are consistent with recent work in humans using cryopreserved decellularized allograft tissue. Elkins et al. recently reported that the humoral immune response to allograft heart valves (measured by PRA) was absent in 52 of 57 (91%) patients at 1 month and was absent in 43 of 49 (88%) at 3 months in allograft valves treated with the SynerGraft process for antigen reduction.[24] Short-term valve function was reported as satisfactory. These findings are particularly relevant given the current research demonstrating that allograft tissue may be sensitizing infants undergoing congenital cardiac surgery,[7,8] infants that may eventually require cardiac transplantation. While the tissues in the current study were valved conduits, it could be expected that decellularization of allograft patches such as those used in the Norwood procedure would have a similarly reduced humoral immune response.

The results of this study confirm that decellularization reduces the immunogenicity of allograft tissue. This finding may in turn be associated with improved

allograft durability. Moreover, the finding of a reduced humoral response may prevent the sensitization which is currently seen with allograft tissue in children, complicating subsequent transplantation. However, the ultimate widespread use of such tissue will be limited by donor availability. Others have investigated the use of decellularized xenograft tissue but this has met with failure[25] presumably due to interspecies extracellular matrix immunogenicity.[26] Use of synthetic matrices seeded with autologous cells has shown promise[27] they may be limited by the need to harvest, expand, and seed autologous cells, a process which may take 14 days or greater. At present, it thus appears that decellularization of allograft tissue may be the most feasible of the three aforementioned options.

One of the major limitations to this study is use of a nonfunctional model which results in sinus of Valsalva thrombosis. One may consider using functional models to overcome such difficulties including larger animal models of pulmonary artery implants or a recently described functional rat model with aortic insufficiency.[28] The current issue of thrombosis makes it difficult to speculate on the effect the reduced cellular infiltrates will have on long-term durability. However, since the cellular infiltrates have been correlated with reduced valve durability, it could be expected that durability would be improved. In addition, longer term studies are required to investigate the capacity for the matrix to repopulate with host endothelial and interstitial cells. Since it could be expected that many of these decellularized allografts would be stored using cryopreservation, the impact of cryopreservation on an acellular matrix needs careful assessment. However, in this experiment we wanted to limit the number of variables which would confound our interpretation of the effects of decellularization. In previous

work with the same rodent model our group had demonstrated that cryopreservation is associated with lack of protection from allorecognition, increased valve leaflet injury, and increased cellular infiltrates, even in a syngeneic model.[29] Lastly, work is required to ensure the integrity of the decellularized graft and the individual components of the extracellular matrix (e.g. glycosaminoglycans) which may be essential for repopulation of the graft by host cells.

CONCLUSION

We have demonstrated that decellularization of aortic valve allografts is associated with a significant reduction in cellular and humoral immune responses to levels seen with nonimmunogenic syngeneic tissue. These findings suggest that the acellular matrix may provide a suitable environment for repopulation by host cells (eg. fibroblasts) which would provide the extracellular matrix with a regenerative capacity typical of native valves, thus improving allograft durability. Moreover, the reduced humoral response may prevent sensitization of recipients of allograft tissue. This would have enormous implications for the survival and quality of life of all valve recipients, especially children. Ongoing studies in a larger animal model of orthotopic implantation will provide information on the thrombogenicity, functional integrity, and capacity for repopulation by host cells.

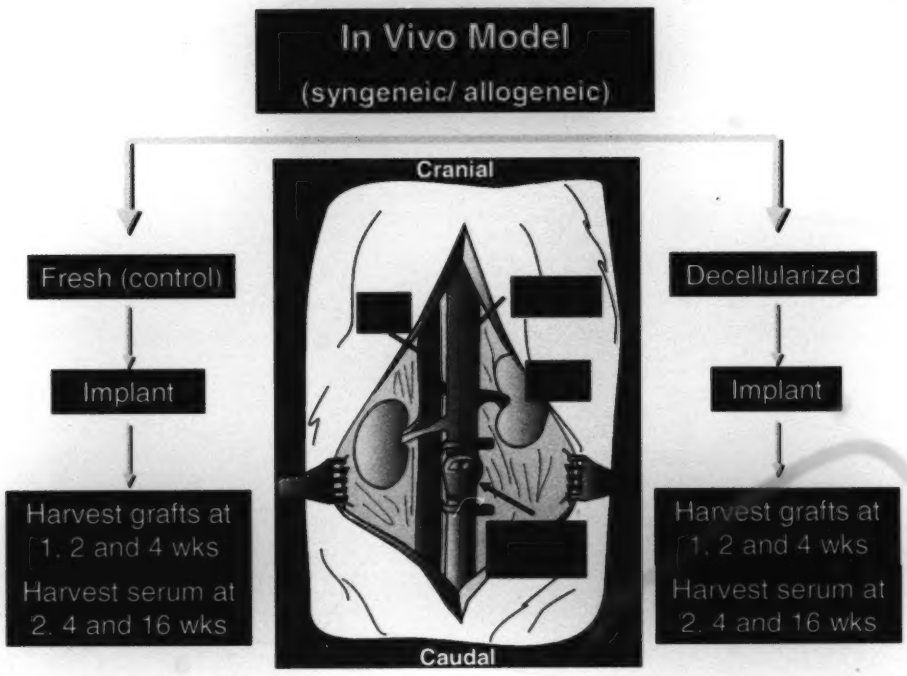

Figure V-1. Study methods, rat aortic valve graft implantation. Aortic valves with approximately 5 mm of ascending aorta were harvested from a donor rat and transplanted into the infrarenal abdominal aorta of recipient rats using end-to-end anastomoses under general anaesthesia. Grafts were implanted as either nondecellularized (fresh) or decellularized grafts in both syngeneic (Lewis donor to Lewis recipient) or allogeneic (Brown Norway donor to Lewis recipient) combinations (n = 6/ time/ group). (Adapted from Yankah et al. Cardiac valve allografts, 1962-1987. Steinkopff Springer; 1988. p. 77-87).

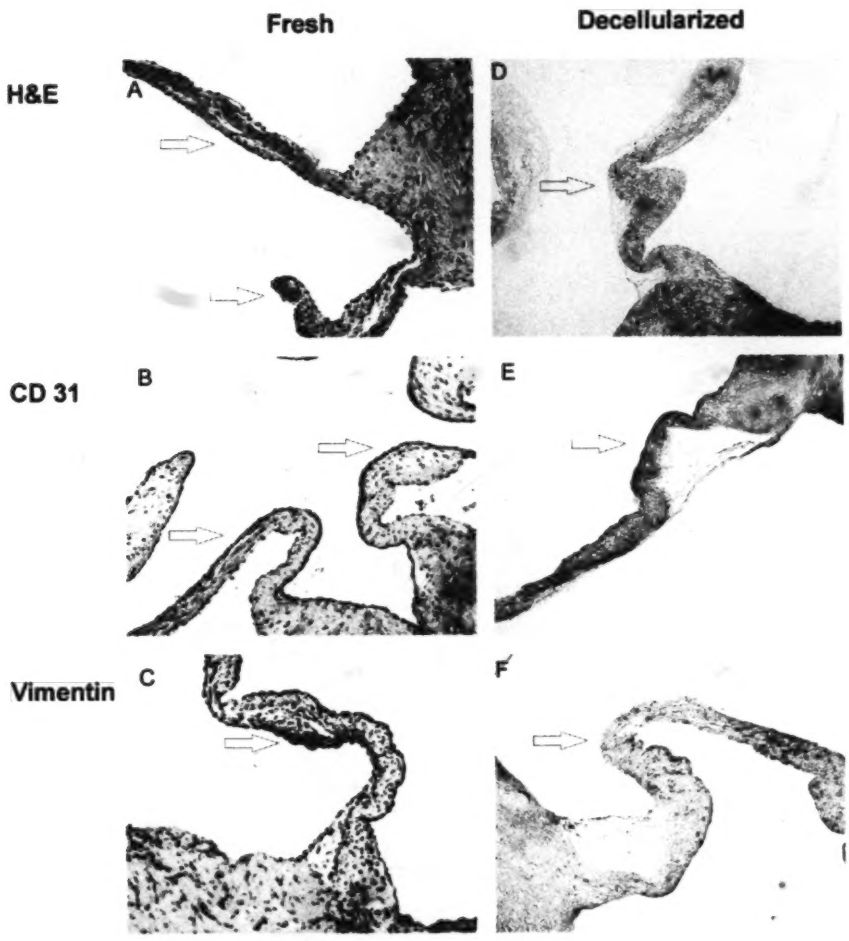

Figure V-2. Representative sections of decellularized grafts prior to implantation. Arrows indicate aortic valve leaflets. H&E staining and immunohistochemistry for endothelial cells (anti-CD31) and interstitial cells (anti-vimentin). Fresh (non-decellularized): note the normal laminar architecture and extensive cellularity with H&E staining. Immunohistochemistry reveals intact endothelial layer, and extensive interstitial cells. Decellularized: preserved extracellular matrix, complete removal of the endothelial layer, and absence of intact cellular elements. Some residual interstitial cellular fragments are noted with anti-vimentin staining. Original magnification 200x.

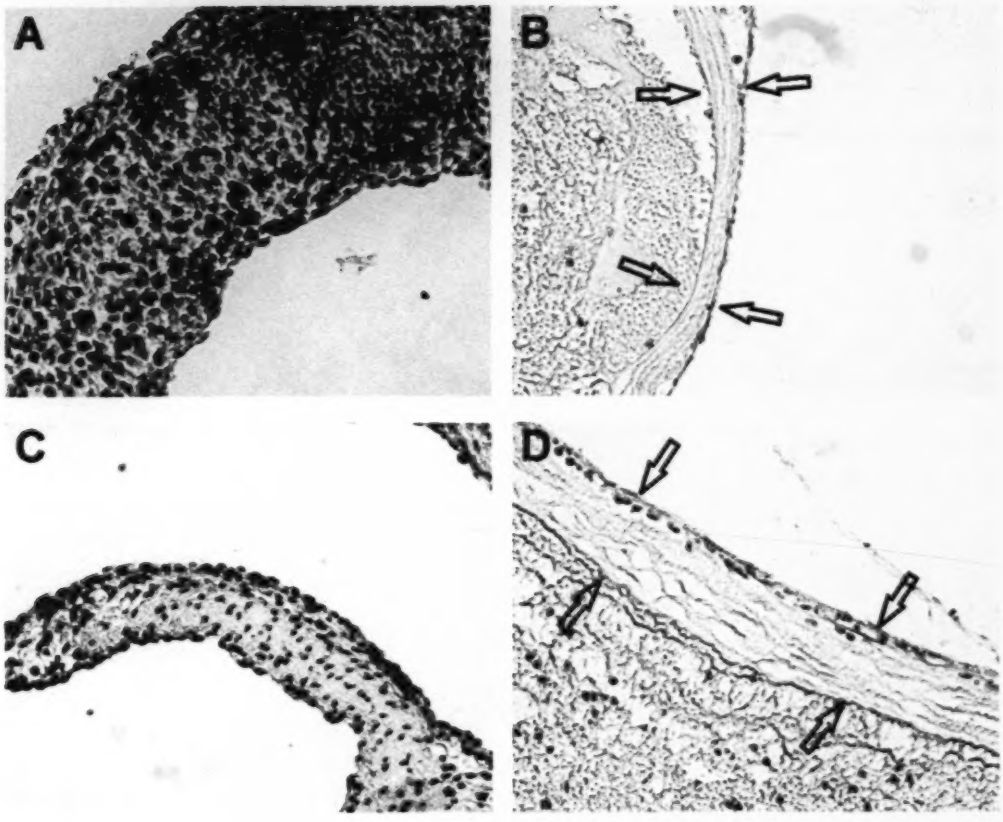

Figure V-3. Representative immunohistochemistry for T cell infiltrates (CD3+) 1 week following transplantation. (A) Allogeneic non decellularized, (B) allogeneic decellularized, (C) syngeneic non decellularized, (D) syngeneic decellularized samples. Untreated allogeneic valve: early and intense T cell infiltrate and significant thickening of the valve leaflets. This was not present in the syngeneic model. Decellularized specimens: in both the allogeneic and syngeneic models there is a dramatic attenuation of the immune response as noted by normal leaflet thickness and the absence of T cells. Arrows indicate location of valve leaflets adjacent to thrombosis in the sinus of Valsalva. Original magnification 400x.

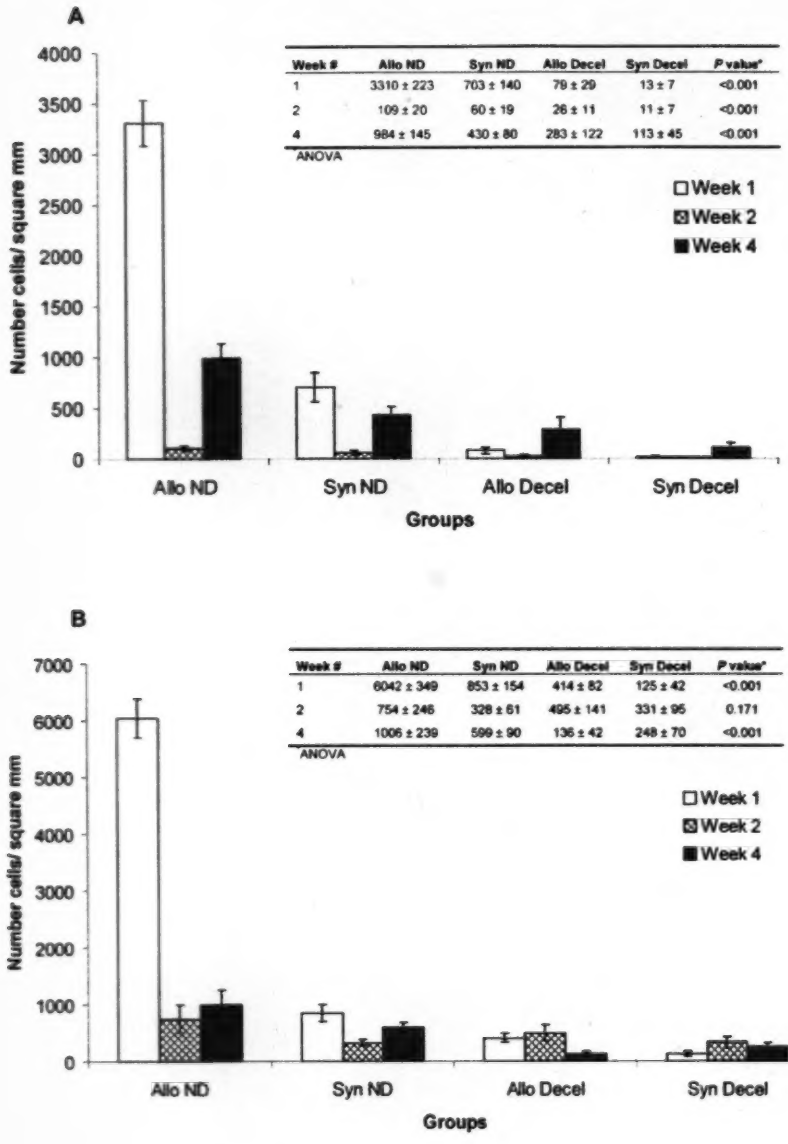

Figure V-4. Summary of morphometric analysis for T cell infiltrates. Decellularization reduces (A) CD3+ and (B) CD8+ mean cell counts in leaflets of aortic valve grafts at 1, 2, 4 weeks post transplantation. (n = 6/ time/ group).

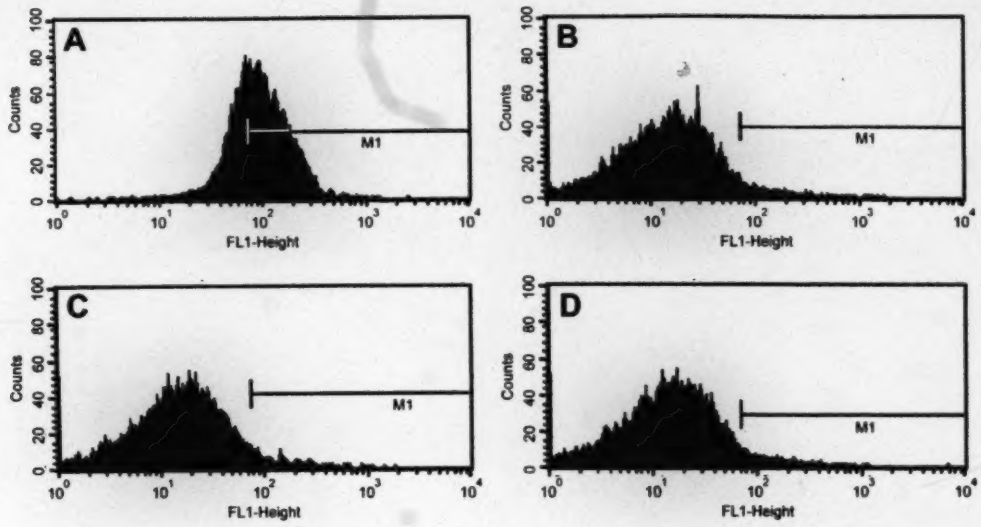

Figure V-5. Representative histograms for flow cytometric analysis at 16 weeks. (A) Allogeneic non decellularized, (B) allogeneic decellularized, (C) syngeneic non decellularized, (D) syngeneic decellularized samples. Allogeneic decellularized tissue was associated with a significant leftward shift indicative of reduced antibody production to donor antigens compared to the nondecellularized allogeneic tissue. Allogeneic nondecellularized tissue had curves which were nearly identical to those of syngeneic tissues.

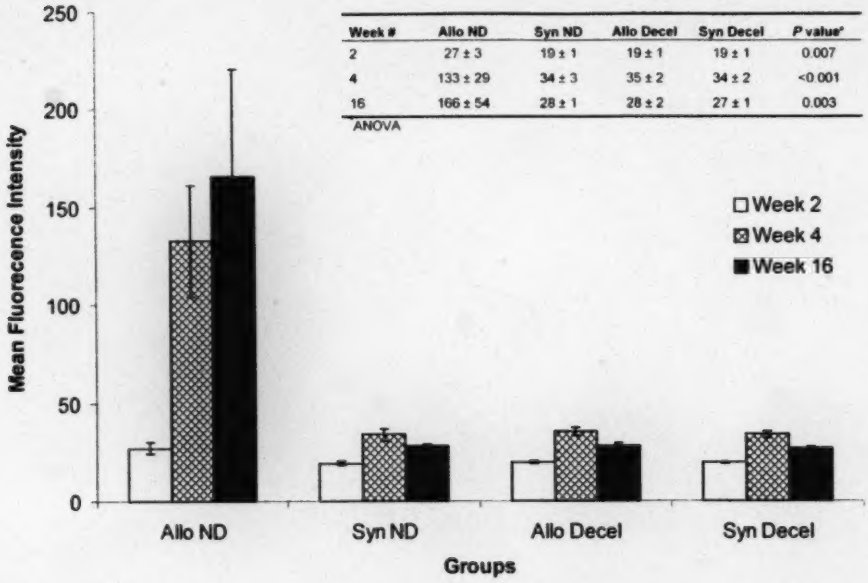
Week # | Allo ND | Syn ND | Allo Decel | Syn Decel | P value*
2 | 27 ± 3 | 19 ± 1 | 19 ± 1 | 19 ± 1 | 0.007
4 | 133 ± 29 | 34 ± 3 | 35 ± 2 | 34 ± 2 | <0.001
16 | 166 ± 54 | 28 ± 1 | 28 ± 2 | 27 ± 1 | 0.003
*ANOVA
Mean Fluorescence Intensity
250
200
150
100
50
0
Week 2
Week 4
Week 16
Allo ND
Syn ND
Allo Decel
Syn Decel
Groups

Figure V-6. Summary of mean fluorescence intensity for flow cytometric crossmatch. Decellularization reduces the humoral response to decellularized allograft valves as identified by flow cytometric analysis at 2, 4, 16 weeks. (n = 6/ time/ group).

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VI

BIOMECHANICAL TESTING OF DECELLULARIZED PORCINE VALVE ALLOGRAFTS

INTRODUCTION

There is increasing evidence that the limited durability[1-3] of cryopreserved allograft tissue used in cardiac surgery is the consequence of alloreactive immune responses.[4,5] In addition to a well-documented cellular immune response to allograft tissue,[6,7] there is evidence for a humoral immune response to this tissue[8,9] which may complicate future transplantation if ever required.[10] While immunosuppression has been shown to reduce the immune response to these tissues,[11,12] typical immunosuppressive agents are not without significant toxicity.[13] Allograft tissues have two main components: the extracellular matrix (ECM) and cells. It has been shown that the cellular elements are the stimulus for the immune response[14] and, therefore, decellularization has been proposed to reduce the immunogenicity of these tissues.[15-17]

We have been investigating a decellularization protocol which utilizes a series of hypertonic and hypotonic buffers to lyse cells, low dose detergent to solubilize membranes, and a washout in isotonic saline solution similar to that of Courtman et al.[18] Investigations in a rodent model have confirmed the efficacy of the aforementioned decellularization technique (submitted) and have demonstrated abrogation of both the cellular and humoral immune response to decellularized allograft.[27] While inbred rodent models provide an invaluable tool to study the immunology of decellularized allograft tissue, they are of insufficient size to study the biomechanical properties of these tissues.

A version of this chapter has been submitted for publication. Nagendran, Carey, McCracken, Lakey, Ross, Meyer. J Heart Valve Dis.

Before proceeding to the ultimate goal of implanting these tissues into humans, it will be essential that the durability of decellularized allograft tissue be confirmed. The logical approach will begin with in vitro testing followed by both long-term in vivo models. The purpose of this study was to determine the biomechanical properties of decellularized porcine aortic and pulmonary valves in vitro.

MATERIALS AND METHODS

Cardiac Tissue Isolation: Whole, fresh porcine hearts were procured from a local abattoir. The pulmonary trunks and aortic walls were excised from the hearts, divided longitudinally into two sections, rinsed with saline solution and stored in Tris buffer (pH 8.0, 50mM, on ice) for transport (<4hrs). One section of the tissue (control; non-decellularized) sample was taken for biomechanical testing immediately and the other section of the tissue samples underwent decellularization before biomechanical testing (n = 6/ group).

Decellularization: The decellularization technique used was similar to that previously described by Courtman et al.[18] Sections of porcine pulmonary trunk and aortic wall were stored in CMRL (90mL, Gibco), FBS (10mL, Sigma) and penicillin-streptomycin solution (penstrep, 0.5mL, Sigma) for 24h at 4°C. Valves were transferred to hypotonic Tris buffer (10mM, pH 8.0) containing phenylmethylsulfonyl fluoride (PMSF, 0.1mM) and ethylenediaminetetraacetic acid (EDTA, 5mM) for 48h at 4°C. Valves were then placed in a hypertonic Tris buffered solution (50 mM, pH 8.0, PMSF 0.1 mM, EDTA 5mM, KCL 1.5 M) containing 1% octylphenoxy polyethoxyethanol (Triton X-100, Sigma) for 48h at 4°C. Valves were then rinsed and placed in Sorensen's buffer (pH 7.3)

containing DNase (25mcg/mL, Sigma), RNase (10mcg/mL, Sigma) and $MgCl_2$ (10mM) for 5h at 37°C. Samples were then transferred to Tris buffer (50mM, pH 9.0, Triton X-100 1%) for 48h at 4°C. Finally, all samples were washed with phosphate buffered saline (PBS) at 4°C for 72 hours, changing the solution every 24 hours. All stages were performed with constant stirring. Valves were stored in Hank's balance salt solution (HBSS) + penstrep at 4°C until time of testing.

Histology: Tissue was formalin-fixed (10%), paraffin embedded, and serially sectioned (5 μm) for histological examination. Efficacy of decellularization and general morphology was assessed by hematoxylin and eosin (H&E) staining.

Biomechanical Testing: Prior to testing, each test strip of porcine pulmonary trunk and aortic wall tissue was allowed to equilibrate for 30 minutes in HBSS (pH 7.4, 310 mOs). The width and thickness of each strip was measured using a non-rotating thickness gauge and recorded. The test strip was mounted between two grips. Each test strip was loaded to fracture on a tensile testing machine (MTS Synergie 500; Eden Prairie, MN) with an extension rate of 10mm/min and the subsequent stress and strain relationship was recorded. Engineering strain was calculated as the change in strip length per unit gauge length while true stress was defined as the force exerted per unit cross-sectional area. Elastic modulus [mmHg/(mm/mm)] was determined from the slope of individual stress-strain curves at low stress (alignment modulus) and high stress (stiffness modulus) and extensibility was determined from the intercept of the high stress elastic modulus (stiffness modulus) tangent and the x-axis of the stress-strain curves similar to that described by Barber et al. (Figure VI-1).[19]

Statistical Analysis: Paired sample testing was used to reduce between-sample variability and statistical analysis was performed using a paired samples t-test. Results were considered significant at $P < 0.05$. Results are expressed as mean ± standard error of the mean (SE).

RESULTS

Representative photomicrographs of aortic wall and pulmonary trunk are provided in Figure VI-2. There was effective removal of cellular elements and preservation of the ECM.

Representative stress-strain curves are provided in Figure VI-3. Decellularization resulted in minimal change in the overall characteristic of the curve for aortic tissue. On the other hand, review of a representative curve for decellularized pulmonary trunk reveals a reduced slope at low stress and a rightward shift suggestive of increased extensibility. Stress to fracture testing demonstrated that the mechanical properties of decellularized cardiac allograft tissue were similar to that of non-decellularized allograft tissue (Figure VI-4). Stress at fracture was similar for decellularized vs. non decellularized aortic wall (7413 ± 567 vs. 7668 ± 549 mm Hg; $P = 0.416$) and pulmonary trunk (10294 ± 883 vs. 10217 ± 963 mm Hg; $P = 0.945$). There was a decrease of under 3% of the total tensile stress to fracture and the tensile stress to fracture of the decellularized tissue greatly exceeds the physiological loading range.

Slopes of the stress strain curves were measured at low stress and high stress. In general, decellularization was associated with a minor and statistically nonsignificant reduction in the elastic modulus (Figure VI-5). There was a minor reduction in the

elasticity of decellularized aorta compared to nondecellularized aorta at low stress (605 ± 29 vs. 698 ± 57 mmHg/ (mm/mm); P = 0.141) and high stress (7058 ± 590 vs. 9276 ± 1600 mmHg/ (mm/mm); P = 0.170), respectively. Similarly, there was a minor reduction in the elasticity of decellularized pulmonary artery compared to nondecellularized pulmonary artery at low stress (396 ± 39 vs. 402 ± 36 mmHg/ (mm/mm); P = 0.897) and high stress (12944 ± 1824 vs. 18902 ± 2817 mmHg/ (mm/mm); P = 0.161), respectively.

Extensibility data is summarized in Figure VI-6. Decellularized aortic wall was significantly more extensible than nondecellularized control tissue (1.84 ± 0.09 vs. 1.39 ± 0.10 mm/mm; P = 0.001). Decellularized pulmonary artery was similarly more extensible than nondecellularized control tissue (2.12 ± 0.16 vs. 1.71 vs. 0.13 mm/mm; P = 0.110) but this value did not reach statistical significance due to slightly greater variability.

DISCUSSION

Many advancements have been made in the field of congenital cardiac surgery and the results similarly continue to improve.[20] Many of the procedures are performed on neonates and infants in the first year of life with correspondingly small hearts and often require the use of prosthetic material to reconstruct on replace abnormal or absent cardiac structures. The options for prosthetic material are limited and requirements are stringent. Options include synthetic, xenograft (porcine/ bovine), or allograft material. Regardless of the material used it must be durable, hemostatic but not thrombogenic, resistant to infection, and must have excellent handling properties in order for procedures to be performed on minute structures. Ideally, this tissue would also be capable of growth. Of

all the materials currently available, allograft tissue most closely fulfills these criteria. Allograft tissue traditionally is harvested from cadavers soon after death, cryopreserved, and stored for future use. In the case of congenital cardiac surgery, this material is used as valved conduits (aortic and pulmonary) to replace valves and as patches to enlarge narrowed arteries and veins. The disadvantage of this material is it is highly immunogenic.[4-6] The consequence of this immunogenicity is the tissue rejects and eventually fails, often in a predictably rapid fashion, especially in young children.[3] Furthermore, this tissue stimulates the generation of anti-HLA antibodies (i.e. sensitization)[8,9] which may complicate subsequent heart transplantation if ever required.[10]

In an effort to abrogate the immune response our group and others have been investigating methods to reduce the immunogenicity of these tissues. Decellularization has been proposed to remove the immunogenic cellular elements. This result is an acellular, non-immunogenic matrix which may be capable of repopulation by host cells. Decellularization protocols, however, represent a balance between two opposing goals: effective removal of immunogenic cellular elements and preservation of biomechanical properties. In a rodent model we have shown that decellularization abrogates the cellular and humoral immune responses.[27] Assessment of the biomechanical properties of decellularized tissue was the objective of the current study. Preservation of these properties will be essential for long term durability and safety.

In this study we demonstrated that our decellularization technique preserves the biomechanical properties of the tissues tested. Differences in stress at fracture between the treatment groups were less than 3 percent for aortic tissue, less than 1 percent for

pulmonary tissue, and did not reach statistical significance. In addition, the stress at fracture values occurred well above physiologic ranges for both aortic and pulmonary tissues. There was a minor reduction in the elastic modulus for both decellularized aorta and pulmonary artery at both low and high stress. The physiologic significance of these differences is unclear. The elastic modulus better approximates the physiologic conditions to which these tissues are exposed with repeated loading and unloading during the cardiac cycle. However, again, these differences were relatively minor and not statistically significant. Lastly, when comparing extensibility data there was only a slight increase in strain suggesting that decellularized tissue is slightly more extensible.

Explanations for the subtle changes in the biomechanical properties of these tissues are unclear but may relate to changes in the constituents, but not necessarily the overall structure, of the ECM. The ECM consists of three main components: 1) insoluble collagen fibres, 2) insoluble elastic fibres, and 3) soluble glycosaminoglycans (GAGs), proteoglycans, and nonfibrillar proteins. The large collagen and elastic fibres are likely unaffected by the decellularization process and thus there is little change in the stress at fracture for decellularized tissue. GAGs on the other hand, play a role in determining the viscoelastic properties of tissues.[21] Abnormalities in GAG content has also been correlated with mechanical abnormalities of valves in a number of disease states including myxomatous mitral valves [22] and anorexigen-induced valve disease.[23] It has been demonstrated by others working with the same protocol that in pericardial tissue that decellularization results in loss of these soluble ECM components.[18] We have noted similar findings in previous experiments (submitted) in which decellularization resulted in increased proportions of collagen per dry weight of tissue. This loss of GAGs and

proteoglycans may explain the subtle changes in the elastic modulus noted in these current studies.

Clinical relevance of this porcine model is confirmed by comparison to similar studies in human tissue which reported similar results.[24,25] Our studies were limited to static testing. Despite measurement of elastic modulus, this form of testing does not directly replicate physiological conditions to which this tissue will be exposed. Dynamic high-strain-rate testing has been advocated as more representative of physiological conditions and more indicative of the viscoelastic properties of these tissues.[26] Such tests include large deformation cyclic loading, stress vibration, and forced vibration. Regardless, such detailed tests still only provide in vitro data and don't completely replicate physiologic conditions. Alternative testing could include in vitro pulse duplicators and or an in vivo model.

CONCLUSION

Decellularized porcine pulmonary trunk and ascending aortic wall had similar mechanical properties to non-decellularized allograft tissue. This suggests that decellularized allograft tissue may be strong enough to be used as a patch in congenital cardiac surgery. However, these tests only approximate physiologic conditions and in vitro studies in large animal models (e.g. sheep) will be essential before translation to use in humans. Regardless, given the accumulating evidence that decellularized allograft tissue is associated with a reduced alloreactive immune response, this could have a significant benefit for children requiring congenital cardiac surgery.

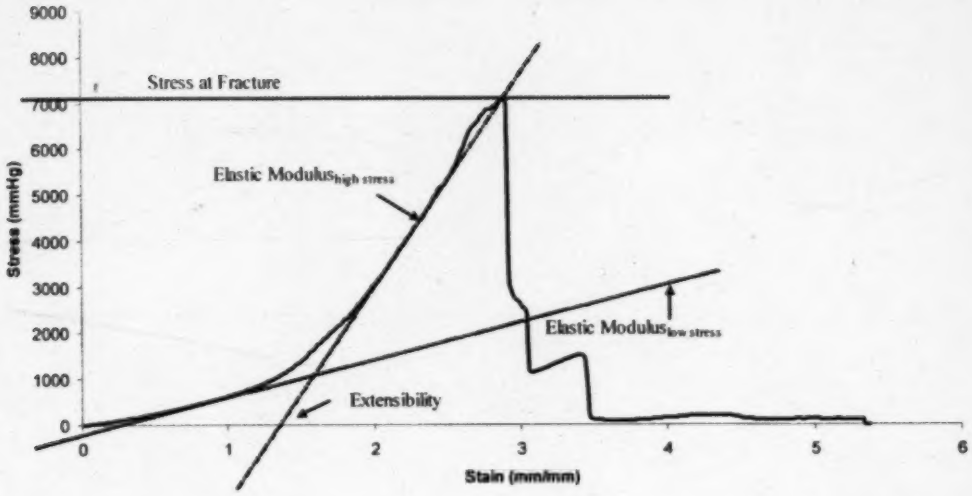

Figure VI-1. Study methods: analysis of stress-strain curves. Stress-strain curves were analyzed to determine stress at fracture, low stress (alignment) and high stress (stiffness) elastic modulus, and extensibility. (Adapted from Barber at al. J Thorac Cardiovasc Surg 2001;122:955-62).

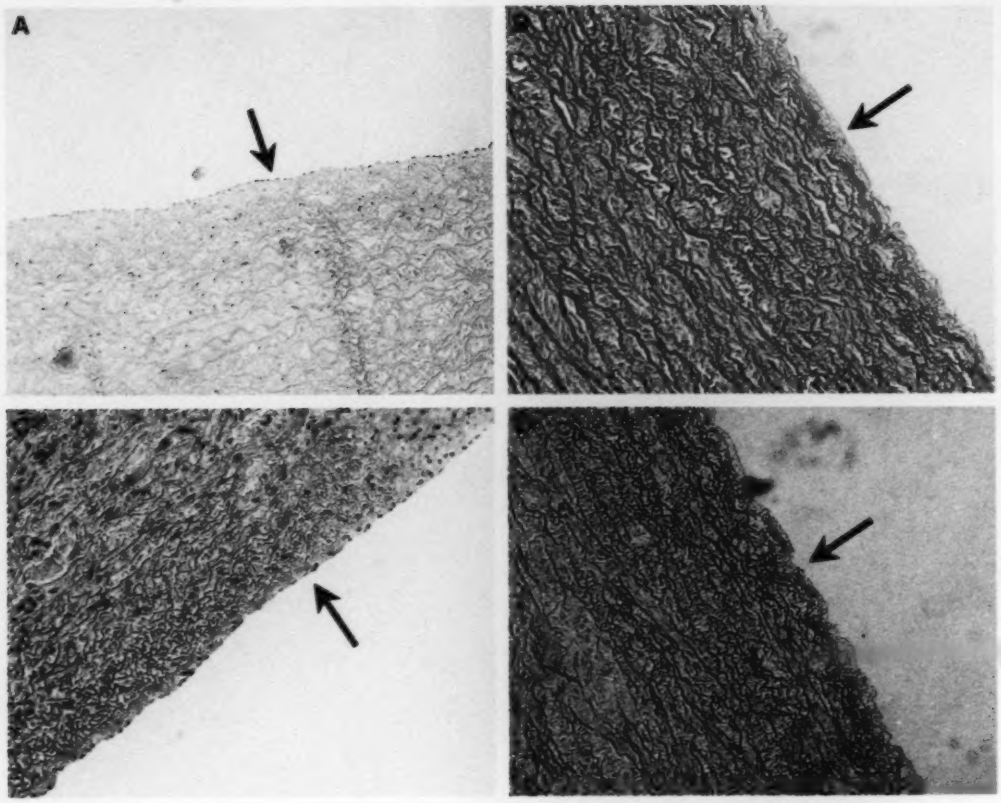

Figure VI-2. Representative histology of control (nondecellularized) and decellularized porcine aortic wall and pulmonary trunk. (A) Aortic wall control, (B) aortic wall decellularized, (C) pulmonary trunk control, (D) pulmonary trunk decellularized stained with hematoxylin and eosin. These photomicrographs demonstrate effective decellularization and gross preservation of the extracellular matrix. Original magnification 200x. Arrows indicate endothelial (luminal) surface.

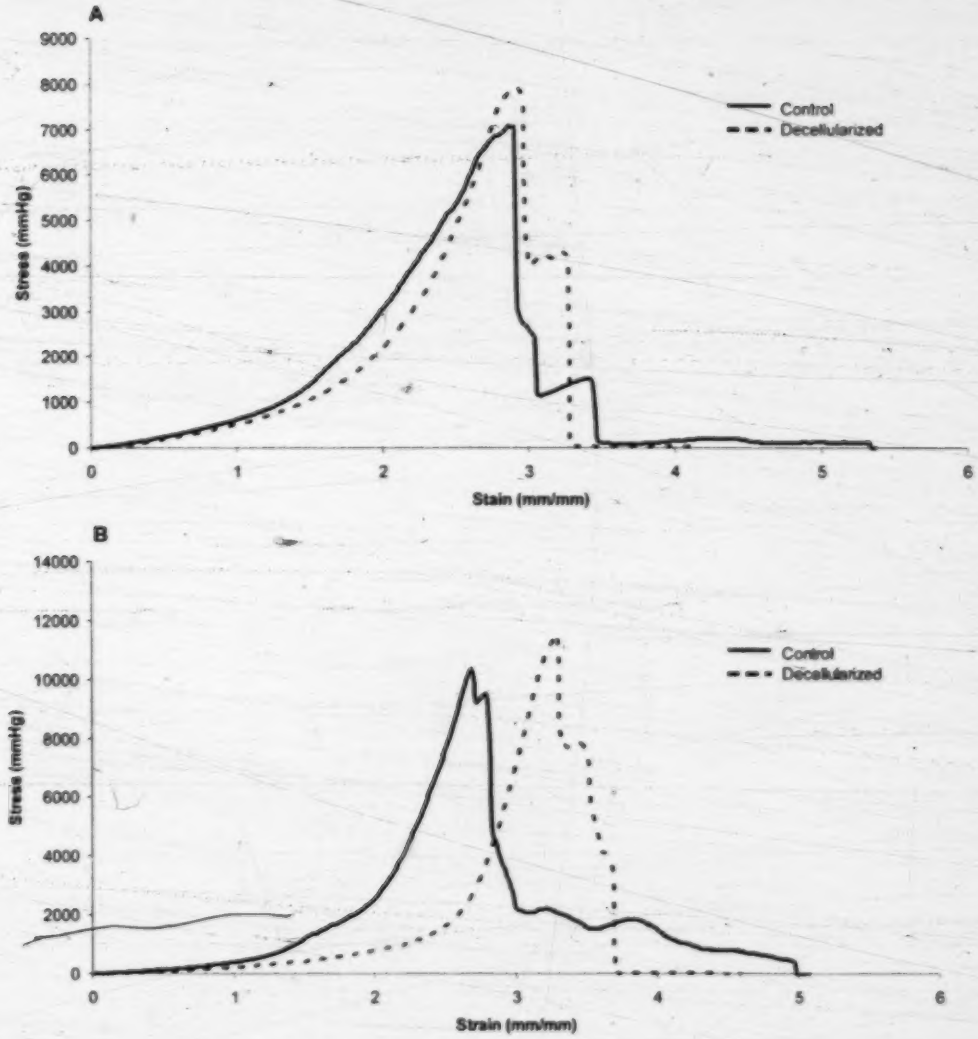

Figure VI-3. Representative stress strain curves for control (nondecellularized) and decellularized porcine aortic wall and pulmonary trunk. Decellularization was associated with minimal changes in the biomechanical properties of (A) aortic wall and modest changes to (B) pulmonary trunk in these sample stress strain curves.

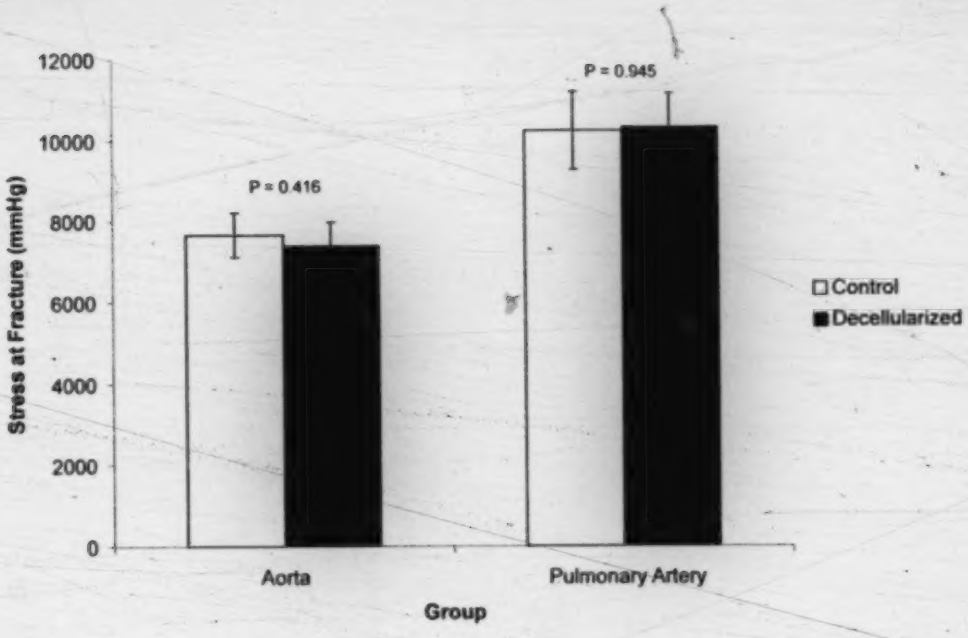

Figure VI-4. Summary of stress at fracture values for control (nondecellularized) and decellularized porcine aortic wall and pulmonary trunk. There was no significant difference between control and decellularized aortic wall or pulmonary artery stress at fracture. (n = 6/ group).

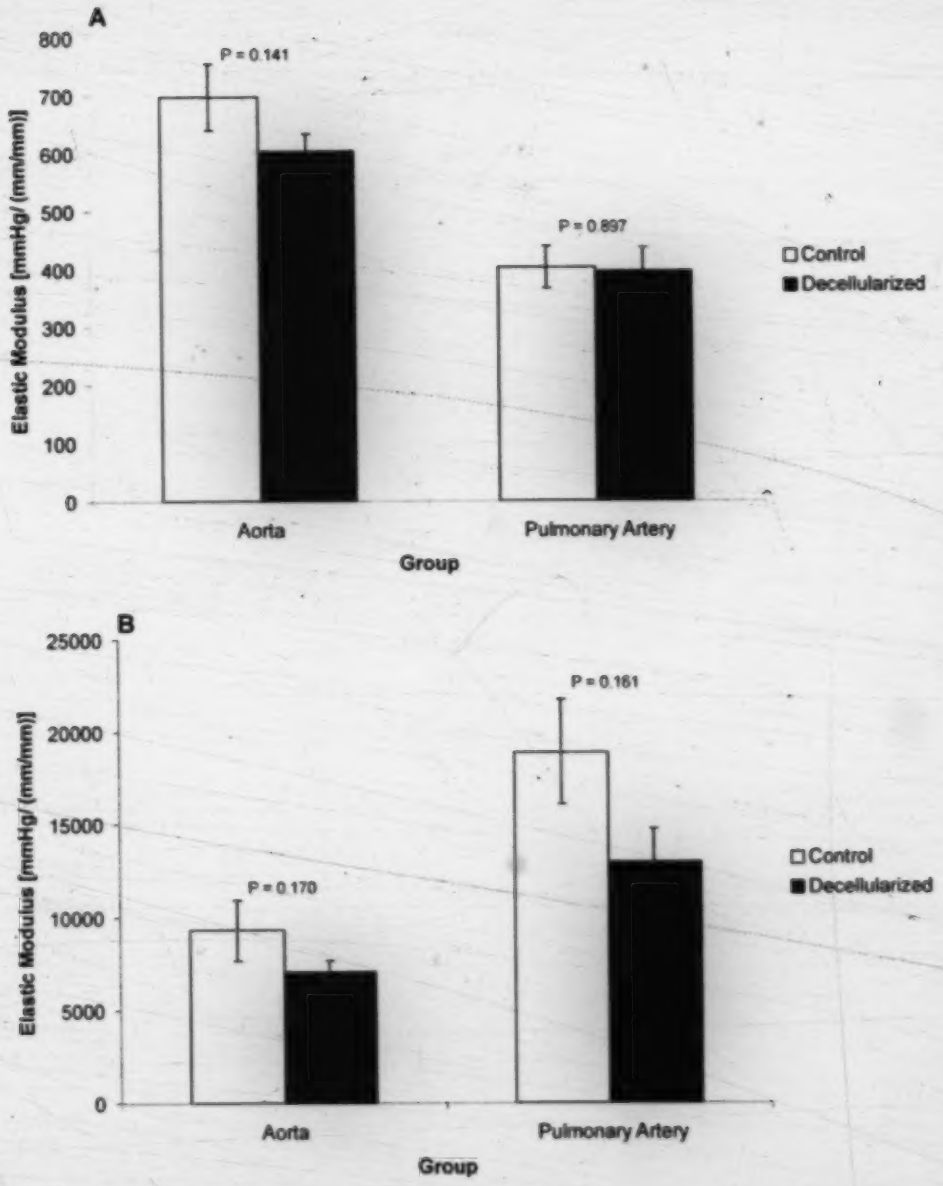

Figure VI-5. Summary of elastic modulus calculations for control (nondecellularized) and decellularized porcine aortic wall and pulmonary trunk. Elastic modulus was calculated from slope of stress-strain curves at low stress (A; alignment modulus) and high stress (B; stiffness modulus). (n = 6/ group).

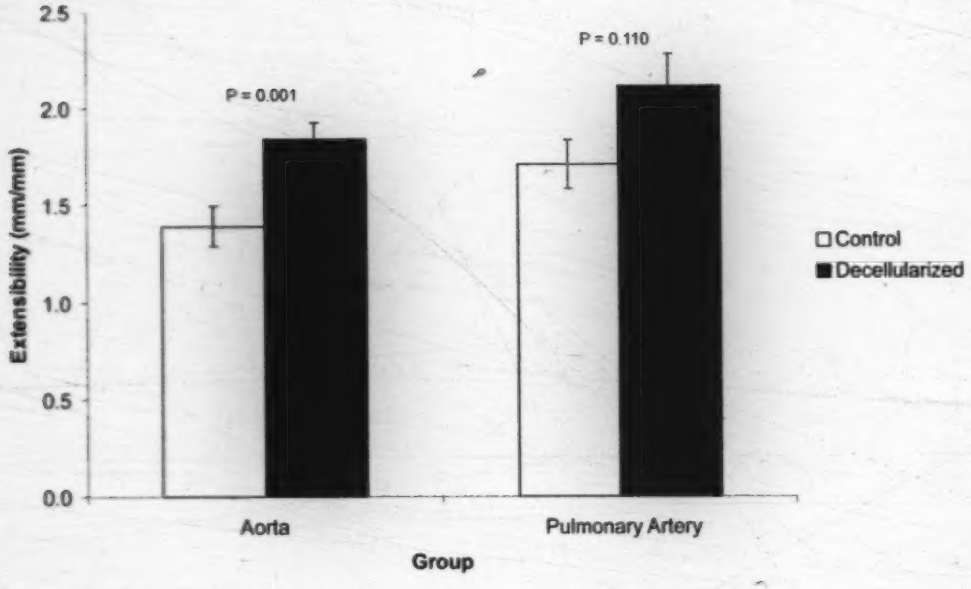

Figure VI-6. Summary of extensibility calculations for control (nondecellularized) and decellularized porcine aortic wall and pulmonary trunk. Extensibility was calculated from the intercept of the high stress elastic modulus (stiffness modulus) tangent and the x axis of the stress-strain curves. (n = 6/ group).

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VII

PROPHYLACTIC INTRAVENOUS IMMUNOGLOBULIN TO PREVENT SENSITIZATION TO CRYOPRESERVED ALLOGRAFT TISSUE USED IN CONGENITAL CARDIAC SURGERY: A PILOT STUDY

INTRODUCTION

It has been well documented that prior sensitization complicates solid organ transplantation. The presence of preformed anti-HLA antibodies, measured as the panel reactive antibody (PRA), is associated with earlier and more frequent high grade rejection,[1,2] increased graft vasculopathy[3], and decreased survival.[4] Importantly, Jacobs et al. recently reported that in pediatric transplantation (median age 130 days), a PRA > 10% was associated with increased 30 day (25%) and longterm (50%) mortality compared with those with a PRA < 10% (8% and 15%, respectively).[5] Moreover, elevated pretransplant panel reactive antibodies (PRA) increase the time on the wait list and complicate perioperative management at the time of transplantation.

Common causes of sensitization includes prior transplantation, pregnancy, and allogeneic blood products. In addition, several case series[6] as well as a prospective cohort study from our institution[7] have demonstrated that the cryopreserved allograft tissue used in congenital cardiac surgery causes donor-specific sensitization with Class I and II PRA values approaching 100%. This is particularly concerning for certain groups of infants such as those with hypoplastic left heart syndrome (HLHS) undergoing the Norwood operation with cryopreserved allograft tissue. While the results of this operation

Final patient follow up to be completed January 2006. Submission for publication to follow.

are improving[8] there is concern that many of these children will eventually require cardiac transplantation.

Two options exist to attenuate the alloreactive immune response: alter the graft or alter the host. Decellularization has been proposed to reduce the immunogenicity of allograft and work in our laboratory has demonstrated that this prevents sensitization in an animal model (in press). However, extensive testing will be required before this tissue is used in humans. The use of typical immunosuppressive agents to prevent sensitization is limited by their long-term toxicity. Experience with kidney and cardiac transplants, on the other hand, has demonstrated that IVIG can produce clinically significant and sustained reductions in anti-HLA antibody titres in individuals who have been *previously* sensitized, in turn allowing for successful transplantation.[9-19] However, there is a paucity of information for the use of IVIG to *prevent* sensitization in previously unsensitized individuals.

Thus, the purpose of this novel pilot study is to determine the efficacy of IVIG in *preventing* the development of anti-HLA antibodies in children undergoing repair of HLHS with allograft tissue.

MATERIALS AND METHODS

Study Design: IVIG 10% (Gammunex, caprylate/ chromatography purified; 2 g/kg) was administered 1 day preoperatively and 3 weeks and 4 months postoperatively in children undergoing surgical repair of HLHS with cryopreserved allograft tissue. PRA for Class I and II antibodies was assessed preoperatively and at 1, 4, 6, and 12 months postoperatively using flow cytometry and compared to historical controls who had

undergone repair of HLHS without IVIG. The study was approved by the local ethics committee for human research and written consent was obtained from patients (parents).

Study cohort: Seven infants with HLHS undergoing first-stage palliation (Norwood procedure) with a cryopreserved pulmonary artery patch. Allograft tissue was provided by comprehensive tissue centres at two Canadian University Hospitals (University of Alberta Hospital, Edmonton, Alberta; Toronto General Hospital, Toronto, Ontario, Canada).

Control cohort: Twelve infants with HLHS who had previously undergone the Norwood procedure with an allograft patch to reconstruct the aortic arch 12 to 18 months prior to the current study. No significant changes had been made in operative or perioperative management during this short interval and thus temporal bias should be limited.

IVIG: IVIG 10% (Gammunex, caprylate/chromatography purified) was administered 1 day preoperatively and 3 weeks and 4 months postoperatively. The dose was 2 g/kg (20 ml/kg). These children are quite volume-sensitive and thus the IVIG was administered while still in hospital (preoperatively, 3 weeks) and at the time of their pre-Glenn procedure cardiac catheterization (4 months); hence, the choice in timing. Otherwise, a separate re-admission would be required specifically for this protocol. IVIG was infused based on established guidelines, being given over at least 8 hours.

Variables: Preoperative variables to ensure similarity between the two groups included age, gender, length and weight. Perioperative factors included duration of crossclamping and cardiopulmonary bypass, use of hypothermic circulatory arrest, inotropic support, blood product exposure (amount and type). Postoperative variables included length of stay in ICU, length of stay in hospital, use of inotropes, and blood product exposure.

Donor and Recipient HLA Typing: Donor and recipient Class I and II HLA typing was tested by molecular methodology. Recipient DNA was purified from whole blood using QIAamp® DNA Blood Mini Kit (Quiagen, Valencia, CA). Donor DNA was purified from bone marrow or ACD stored blood. HLA A, B, and DR antigen typing was performed using the low resolution Micro SSP™ DNA typing kit (One Lambda Inc., Canoga Park, CA). DNA fragments were separated by agarose gel electrophoresis. HLA antigens were determined through a combination of One Lambda DNA/LMT software analysis and manual interpretation of the electrophoresis results.

HLA antibody analysis: Screening for anti-HLA antibodies was performed using the Flow PRA® Screening Test (One Lambda Inc.). Serum samples were analysed according to the manufacturer's recommendations. Test control sera included a negative control from One Lambda Inc. (Catalogue number FL-NC) as well as a positive control which was a 1/32 dilution of a local positive pool made from many high PRA patient sera. A 10 µL mixture of class I and class II beads as well as control beads were added to every tube. Patient sera (20 µL) were added and the tubes were incubated for 30 minutes. The tubes were washed twice and 100 µL of diluted FITC conjugate (anti human $F(ab')_2$) was added. A final wash step was performed and the beads were analysed using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). Samples that tested positive for the presence of either Class I and/or Class II antibodies were then further tested for specificities using the FlowPRA® Specific Antibody Detection Test kit (One Lambda Inc). Specificity analysis was also performed by the use of single antigen beads if the PRA > 60% (catalogue numbers FL2HD and FL1HD, One Lambda Inc.). In a few

cases antibody specificity for class II was also done by ELISA methodology. The ELISA kit used was Quickscreen™ Elisa (GTI, Waukesha, WI).

Data analysis/ statistics: All outcomes were expressed as means and standard deviation. Comparisons between continuous data were made with Mann-Whitney U test and comparisons between nominal data were made with Chi-square or Fisher's exact test where appropriate. Differences were considered significant for a value of $P < 0.05$. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS, version 13).

RESULTS

Patient demographics are summarized in Table VII-1. Except for the IVIG group being somewhat older (8.2 ± 4.8 vs. 17.1 ± 14.6 days; P = 0.021), the two groups were well matched preoperatively. The majority of this discrepancy in age resulted from one infant in the IVIG having surgery delayed until 49 days of age. Cardiopulmonary bypass time was similar for the two groups (127.2 ± 59.7 vs. 123.4 ± 40.0 minutes; P = 0.899) but the IVIG group required a marginally shorter circulatory arrest time (28.0 ± 10.6 vs. 18.4 ± 10.9 minutes; P = 0.062). Both groups required similar amounts of blood products perioperatively, including packed red blood cells (12.3 ± 9.6 vs. 10.6 ± 5.0 units; P < 0.966), platelets (4.8 ± 7.5 vs. 3.7 ± 4.6; P = 1.000), fresh frozen plasma (1.2 ± 1.2 vs. 1.6 ± 1.0 units; P = 0.565), and cryoprecipitate (3.8 ± 4.3 vs. 3.9 ± 3.2 units; P = 0.567).

Timing of doses of IVIG administered is summarized in Figure VII-1. The preoperative dose was consistently given at 3.0 ± 2.4 days before surgery, the second dose at 22.4 ± 2.8 days after surgery, and the third dose during hospitalization for the

children's Glenn procedure (141.3 ± 28.3 days after the initial surgery). IVIG was administered without adverse events.

Compared to control infants who did not receive IVIG, there were no significant differences in Class I or Class II PRA values for those who did receive IVIG (Figures VII-2, 3). Preoperatively (prior to the initial dose of IVIG) there were minor elevations in both Class I and II antibodies most likely reflecting maternally transmitted (i.e. passive) antibodies. One month postoperatively (one week after the second dose of IVIG) there was evidence for a humoral immune response with modest elevations in PRA for both Class I (19.7 ± 30.1 vs. 4.5 ± 9.0; P = 0.443) and Class II (17.1 ± 27.5 vs. 20.0 ± 17.1; P = 0.400) antibodies in both the control and IVIG groups. Similar findings were noted at four months for the control and IVIG groups: PRA for Class I (61.9 ± 39.9 vs. 72.7 ± 41.1; P = 0.813) and Class II (49.3 ± 41.9 vs. 53.8 ± 40.5; P = 0.706) were not significantly different. In a more detailed review of the individual PRAs at 4 months for recipients of IVIG reveals that there were nonresponders to IVIG with PRAs approaching 100% and others who responded with PRAs ranging from 0% to 29% (Figure VII-4). HLA typing of donor and recipient confirmed that responders were mismatched for both Class I and II antigens (Table VII-2).

DISCUSSION

The Norwood operation has become the accepted standard of care for newborns with HLHS. This operation requires the use of an allograft cryopreserved pulmonary artery patch to reconstruct the aorta. Despite previous beliefs that this tissue was immunoprivileged, recent investigations by our group and others have provided evidence

that allograft tissues stimulate alloreactive immune responses. Clinical studies by Baskett et al. correlated enhanced viability of aortic valve allografts with increased antigenicity and increased rate of valve failure.[20,21] Other studies in humans and animals have demonstrated an increase in donor-specific cytotoxic[22,23] and helper T-lymphocyte precursors.[24] Using a rat model, our group,[25,26] and others,[27,28] have demonstrated an early and intense cytotoxic T-lymphocyte response along with complete destruction of valve leaflets in allogeneic rats. In addition to the aforementioned cell-mediated response, others have demonstrated a humoral immune response in recipients of allograft tissue.[29-31] Hoekstra et al. reported that panel reactive antibodies developed in 78% of 32 recipients of cardiac valve allografts.[32] Smith et al. similarly noted a strong donor HLA-specific antibody response with HLA antibodies detected in 56% of recipients of antibiotic-preserved allografts and 100% of homovital (fresh) allograft recipients.[33] Hawkins et al. identified a significant increase in alloreactive antibodies in 24 children receiving cryopreserved allografts: at 3.3 months after operation panel reactive antibodies had risen to 92% (1.9% preop) and Class II antibodies had risen to70%.[6] In a prospective cohort study at our institution comparing the PRA of infants receiving allograft tissue during the Norwood procedure to infants undergoing an arterial switch procedure and not receiving allograft tissue we clearly demonstrated that children receiving allograft tissue developed panel reactive antibodies approaching 100%.[7] Moreover, a substantial proportion of the antibodies were donor-specific.

While the results of the Norwood operation for HLHS are steadily improving,[8] it is felt that many of these children will eventually require cardiac transplantation. There is concern regarding the impact the aforementioned sensitization will have on successful

transplantation. It has been documented that the presence of antibodies in the serum of the allograft recipient significantly increase the risk of early allograft failure and poorer patient survival as a result of humoral rejection.[34-38] Particularly notable is an analysis from the United Network of Organ Sharing Registry. In a review of 14 535 heart transplants performed between 1987 and 1996, it was demonstrated that an elevated PRA at transplantation significantly increased the relative risk of graft failure ($P = 0.0001$).[4] Moreover, a PRA > 60% was found to be associated with a 2.242 relative risk of graft failure. Such PRA levels are not uncommon in our HLHS population after receiving allograft tissue in the Norwood operation. Itescu et al. reported that pretransplantation anti Class II antibodies were associated with early development of high grade cellular rejection ($P < 0.0001$) and increased annual rejection frequency ($P < 0.001$).[1] Similarly, Leech et al. reported evidence of acute or hyperacute rejection on endomyocardial biopsy at postoperative day seven in three of four patients who received orthotopic heart transplants despite being highly sensitized.[39]

As more sensitive methods for detecting antibodies have become available, the impact of allosensitization has become even more apparent. Using the more sensitive ELISA method of PRA-STAT, Kerman et al. reported that pretransplant PRA-STAT sera > 10% were at increased risk for graft rejection ($P < 0.05$), more rejection episodes/recipient ($P < 0.02$), and graft rejection within 30 days ($P < 0.001$).[3] The increased sensitivity of the FlowPRA technique was recently reported by Tambur et al.[2] When compared to the CDC method, FlowPRA detected a pretransplant PRA > 10% in 34.8% of the patients who initially tested negative by CDC methodology. Moreover, pretransplant antibodies detected by Flow PRA were highly associated with rejection

episodes ($P < 0.001$) and one-year graft survival ($P < 0.004$). Consequently, the presence of anti-HLA antibodies limits the ability to find a T cell crossmatch negative donor, waiting times for a suitable allograft are considerably longer, and the mortality whilst waiting for a donor is high.

The mechanism by which preformed antibodies cause rejection has been receiving increasing interest. Despite previous documentation of anti-HLA antibodies in recipients of solid organ transplants, it wasn't until more recently that reliable histologic methods became available to document and study this phenomenon. A recent working group recommended term antibody mediated rejection (AMR) to describe rejection involving donor-specific antibodies to HLA antigens, ABO isoagglutinins, and antiendothelial antibodies.[40] AMR can be further classified based on time course. Hyperacute AMR causes graft dysfunction and loss within the first 24 hours of transplantation and acute AMR occurs more than 24 hours after surgery. More important, however, is the recognition of the more indolent process termed chronic AMR and its role in allograft vasculopathy.

Recent technological advancements have been critical to the understanding and diagnosis of AMR. These include ELISA[41,42] and flow cytometry[43] techniques to identify donor specific antibodies and techniques to identify complement activation with C4d staining.[44,45] A recent consensus conference defined the criteria for acute cardiac AMR; this definition includes clinical (graft dysfunction), pathologic (endothelial swelling or denudation, macrophages in capillaries, neutrophils in capillaries, interstitial edema or hemorrhage), serologic (donor-specific antibodies), and immunopathologic (immunoglobulin and C3d or C4d deposition in capillaries) criteria.[46] Despite the

aforementioned relationship between anti-HLA antibodies and graft vasculopathy,[3] a definition for chronic cardiac AMR has yet to be developed by this working group.

Despite extensive investigation, alternative materials to allograft tissue have yet to be identified. Glutaraldehyde-treated xenograft tissue tends to undergo rapid calcification and fail even more rapidly than allograft tissue, especially in children.[47,48] Synthetic (eg. Goretex, Dacron) material tends to thrombose, especially in neonates in which 3-5mm conduits are being used. Moreover, no material comes close to the handling properties of allograft tissue, for instance when attempting to reconstruct the aortic arch of a newborn (3kg child). Thus, allograft tissue continues to play an essential role in congenital cardiac surgery. Numerous potential methods exist or are being investigated to reduce the immunogenicity of allograft tissues. Tissue matching (donor-recipient HLA matching) would be a complex and expensive process: tissue banks would have to have a large number of tissues on hand in order to be likely to provide the correct tissue matched for HLA loci. Decellularization techniques are currently being investigated in a number of laboratories including our own. However, this requires extensive additional investigation.

Altering the host with typical immunosuppressive agents (cyclosporine, mycophenolate mofetil) is effective but is limited by their short- and long-term toxicity.[49] A recent study by Shaddy et al. demonstrated that mycophenolate mofetil (600 mg/m^2/dose) twice daily for 3 months substantially reduces anti-Class I antibodies (but not Class II).[50] One patient withdrew after 2 weeks due to a sinus infection that was successfully treated with oral antibiotics, and 3 patients had a transient adverse effect of postoperative vomiting. Side effects of immunosuppression range from mild (gastritis, nausea, and vomiting, diarrhea) to severe (leukopenia, thrombocytopenia, neutropenia,

and anemia, post-transplant lymphoproliferative disorder, nephrotoxicity, diabetes mellitus, hypertension, hyperlidemia). Thus, despite efficacy, long-term use of these agents in children is difficult to justify.

IVIG is an alternative immunomodulatory agent which has demonstrated to be safe. Experience with kidney transplants and cardiac transplants has demonstrated that this agent can produce clinically significant and sustained reductions in anti-HLA antibody titers in individuals who have been previously sensitized, in turn allowing for successful transplantation.[9-19] Pisani et al treated 16 highly sensitized adult patients with plasmapheresis and IVIG (20 g/patient) prior to transplantation. When compared to a cohort without elevated pretransplant antibody levels, there was no difference in early outcomes, survival, or rejection.[17] John et al. reported a 33% reduction ($P < 0.01$) in PRA levels with IVIG with 1 to 3 monthly courses of IVIG (2 g/kg), in turn allowing for earlier cardiac transplantation of highly sensitized individuals.[18] A recent paper by Glotz et al. reported the successful desensitization in 13 of 15 (87%) patients in a pilot trial with 3 monthly courses of 2 g/kg body weight IVIG.[19] These 13 patients underwent immediate kidney transplantation, with loss of only one of the kidneys to rejection. Lastly, the NIH-sponsored IG02 trial randomized 101 adult end-stage renal disease patients with PRA > 50% to IVIG (2 g/kg monthly for 4 months) or placebo.[51] IVIG therapy was associated with a modest improvement in transplantation rates (35% vs. 20%; $P = 0.069$) on intention-to-treat analysis. IVIG was also associated with reduced time to transplantation ($P < 0.05$) and decreased mortality (8% vs. 16%; $P = 0.22$). These findings of increased transplantability occurred despite only a modest reduction in IgG

PRA values (absolute reduction < 20%; P = 0.007 on repeated measures analysis) and failure to reduce PRA levels to < 40%.

There are numerous mechanisms by which IVIG is thought to exerts its immunomodulatory effects.[52-54] In general, these mechanisms involve the various components of the immunoglobulin molecule including the variable $F(ab')_2$ region, the Fc region which interacts with Fc receptors (FcR), the Fc region which interacts with complement factors, and immunomodulatory substances other than immunoglobulin in IVIG.[55] More specifically, neutralization of autoantibodies by anti-idiotypic antibodies has been shown to be an important mechanism action of IVIG in autoimmune diseases.[56-58] Similarly, neutralization of alloantibodies by anti-idiotypic antibodies may be a mechanism by which IVIG reduces PRA.[9,52,59] There is evidence that IVIG significantly inhibits expression of the coreceptor CD19 on activated B cells.[60] IVIG has also been shown to modulate B cells through the inhibitory receptor FcγRIIB pathway.[61] This has the potential for long-term elimination of HLA-specific B cell lines. IVIG also modulates the complement system. Inhibition of C3b/ C4b results in reduced stimulation of complement receptors (CR) 1, 2 (and thus T cell stimulation) and decreased CD19 stimulation (and thus B cell stimulation).[52,62] IVIG has also been shown to inhibit MAC (membrane attack complex) formation and thus decrease antibody-mediated cell injury. Other effects include inhibition of T cell-antigen presenting cell interaction by solubilized membrane components and HLA determinants; reduced production of the cytokines IL 1,2,4,6; inhibition of helper T cell proliferation;[63] and increased suppressor T cell activity. Finally, the immunomodulatory role of natural antibodies has been receiving increasing attention. [54,64]

In this study we tested the hypothesis that IVIG could prevent sensitization in previously unsensitized individuals. Despite the extensive aforementioned documentation of the effectiveness of IVIG in reducing alloreactive antibody levels, this pilot study did not demonstrate any benefit in giving IVIG in advance of exposure to allogeneic material. In the 7 patients in this study, there was no difference in the PRA of those receiving IVIG compared to those not receiving IVIG. Paradoxically, the IVIG group had slightly higher PRA. The reason for these elevated PRAs is not clear. It may be argued that the immunoglobulins in IVIG are being detected by the PRA analysis; however, the Flow PRA method and the subsequent specificity analysis is specific enough to differentiate IVIG vs. host-generated antibodies (personal communication). The lack of response to IVIG may be dose-related; however, we used a dose (2 g/kg) that is standard in most successful desensitization protocols including the NIH IG02 trial.[16,18,19,51] Moreover, it is important to note that our study did not utilize additional desensitization techniques including plasmapheresis and other immunosuppressives noted in many of the previous case series.

The dosing regimen took into consideration the half-life of IVIG (3weeks) and the need to administer the agent under closely monitored conditions in these very volume-sensitive infants. Hence, the reason for choosing the dosing times: preoperative (prior to exposure to allograft tissue), 3 weeks (infant still in hospital), 4 months (infants returns for Glenn procedure). The Stollery Children's Hospital is a tertiary referral centre for pediatric heart surgery and receives patients from an extremely large geographic area (Western Canada) and thus having infants return for more frequent dosing would not have been possible. Consequently, the major limitation of this study was the need to administer

the IVIG while in hospital and the resultant inability to administer IVIG at 2 and 3 months after surgery. This time period is the critical window of antibody development when the child is most likely developing memory B cells to the allograft tissue. Thus, in effect, we were only testing the efficacy of the first two doses of IVIG and the impact of the third dose at 4 months is questionable. Regardless, Glotz et al. achieved a 50% reduction in PRA with 3 monthly courses of 2 g/kg IVIG.[19] John et al. reported a mean reduction of 33% in anti-HLA Class I reactivity within 1 week of 2 g/kg IVIG.[18] Moreover, Glotz et al. demonstrated that the maximal reduction in alloreactivity occurs within 1 week of IVIG therapy and that sequential doses of IVIG did not have an additive effect on reduction of circulating anti-HLA Class I IgG antibodies. Similarly, in the IG02 trial which administered IVIG 2 g/kg monthly for 4 months, the majority of the reduction in PRA was seen within the first month.[51] The findings of these studies contrast sharply with ours in which there was no response to IVIG seen within the first month of therapy.

This study differed from all prior trials of IVIG therapy in a number of key factors. First of all, our patient population was neonates and infants who have very different immune systems compared to those of adults. There are well-documented differences in the T-independent humoral immune responses of neonates.[65,66] Perhaps there are subtle differences in the T-dependent humoral immune response which abrogate the immunomodulatory effects of IVIG. Secondly, the majority of the studies reporting desensitization with IVIG do so in patients who have had remote sensitizing events and are not currently being exposed to foreign antigens. In contrast, our study involved concurrent exposure to both foreign antigens and IVIG. Perhaps the presence of an ongoing antigenic stimulus overwhelmed the immunomodulatory effects of IVIG. This

hypothesis is complicated by reports of IVIG reversing acute humoral rejection after solid organ transplantation in adults.[14,15]

Our study has a number of limitations. Small sample size limits statistical calculations. However, reviewing the scatterplots and comparison to control patients who did not receive IVIG demonstrates a lack of response to IVIG. There is increasing discussion that the PRA may not be the most suitable test to determine sensitivity.[40] Antibody specificity and titers may be a more sensitive method to determine the degree of sensitization. Alternatively, using less sensitive methods than Flow PRA such as complement dependent cytotoxicity (CDC) and antiglobulin-enhanced CDC (AHG-CDC) may have provided additional information. Additionally, longer follow up may reveal a delayed effect of IVIG after 4 months. Finally, if heart transplantation is required in the future, determination of crossmatch results may be revealing.

CONCLUSION

In conclusion, this novel study has been unable to demonstrate that 2 doses of IVIG prevents sensitization at 4 months after exposure to allograft tissue in neonates undergoing congenital cardiac surgery. These findings occur despite studies in adults which have demonstrated that high dose (2 g/kg) IVIG reduces sensitization. Additional follow up after the third (4 month) dose is required. The ultimate test will be long term follow up and determination if early treatment with IVIG improves future transplantability.

Table VII-1. IVIG Patient Demographics

Variable[1]	No IVIG	IVIG used	P-value
N	12	7	
Age at surgery (days)	8.2 ± 4.8	17.1 ± 14.6	0.021
Gender (M%)	75%	71%	1.0
Length (cm)	50.5 ± 2.4	51.3 ± 3.4	0.608
Weight (kg)	3.4 ± 0.4	3.3 ± 0.5	0.734
XC time (min)	42.6 ± 27.7	31.1 ± 17.9	0.310
CPB time (min)	127.2 ± 59.7	123.4 ± 40.0	0.899
TCA time (min)	28.0 ± 10.6	18.4 ± 10.9	0.062
PRBC (units)	12.3 ± 9.6	10.6 ± 5.0	0.966
Platelets (units)	4.8 ± 7.5	3.7 ± 4.6	1.000
FFP (units)	1.2 ± 1.2	1.6 ± 1.0	0.565
Cryo (units)	3.8 ± 4.3	3.9 ± 3.2	0.567

[1]XC time, cross clamp time; CPB time cardiopulmonary bypass time; TCA time, total circulatory arrest time; PRBC, packed red blood cells; FFP, fresh frozen plasma; Cryo, cryoprecipitate

Table VII-2. Donor-recipient HLA antigen mismatch and antibody specificities at four months.

Pt	Antigen Mismatches [1,2]		PRA 4 months		Antibody Specificities	Donor Specific Antibodies
	Class I	Class II	Class I	Class II		
1.	A1 A24 B8	DR17 DR15 DR52	0	0	NA; DR-PENDING	NA
2.	A3 B7 B62	DR11 DR16 DR51 DR52	98	76	A2 A3 A30 A68 A11 A34 A24 A32 A33 A31 A23 A13 B35 B62 B45 B60 B44 B49 B57 B7 B8 B55 B65 DR11DR13 DR14 DR15 DR16 DR17 DR18 DR52 DR4 DR5 DR6 DR8 DR9	A3 B7 B62 DR16 DR11 DR52
3.	A1 A30 B18 B63	DR1 DR13	96	29	A1 A3 A29 A30 A26 A11 A32 A31 B18 B62 B57 B52 B8 DR1 DR103 DR16	A1 A30 B18 DR1
4.	NA	NA	98	93	A 23 A24 A29 B49 B13 B45 B60 B44 B57 B7 B8 B55 DR4 DR7 DR13	NA
5.	A2 B44 B62	DR4	98	98	A1A2 A68 A25 A26 A34 A32 A23 A24 A29 A11 B 49 B51 B52 B62 B35 B57 B44 B45 B65	A2, B44, B62, CLASS2 PEND
6.	NA	NA	46	27	?A25 ?A26 ?A66 DR1 DR10	NA
7.	A32 B27	DR17 DR103	N/A	N/A	N/A	N/A

[1] Antigen mismatches: donor antigens at which recipient was not matched

[2] N/A: donor or recipient typing was not performed

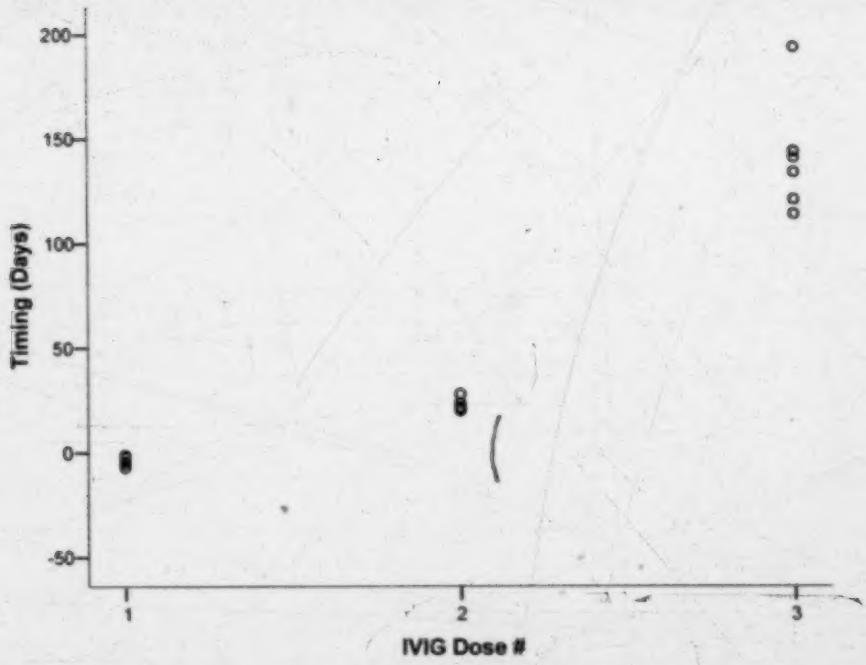

Figure VII-1. Scatter plot of timing of IVIG administration. The first and second dose of IVIG was consistently given at the indicated time. The timing of the third dose was more variable due to the requirement for IVIG to be given while the patient was in hospital undergoing other procedures.

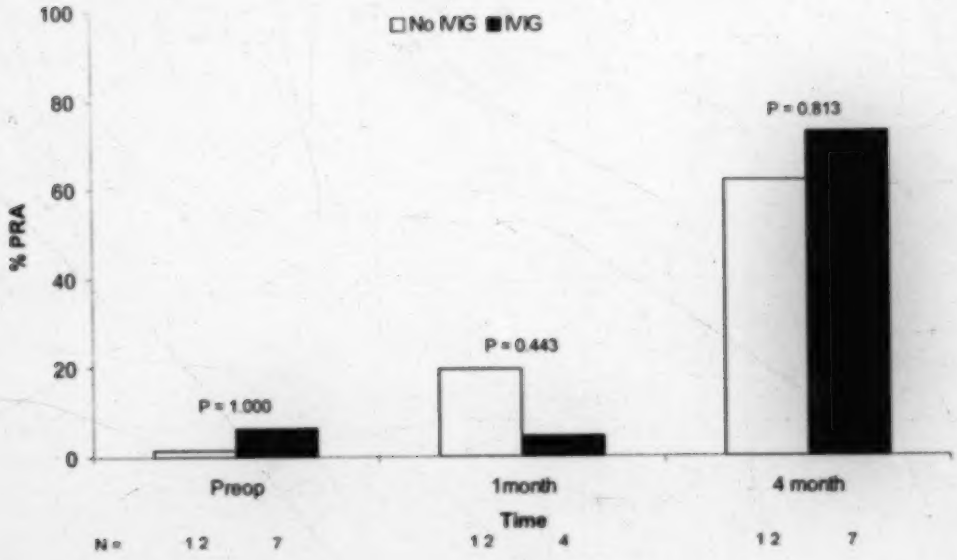

Figure VII-2. Class I PRA levels: preoperative and 1, 4 months postoperatively.

IVIG did not decrease anti-HLA Class I antibody generation.

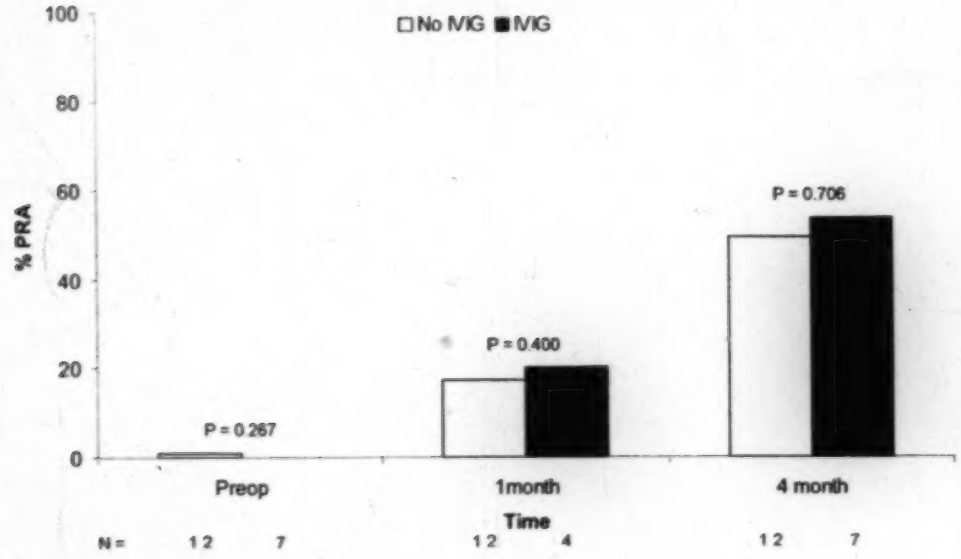

Figure VII-3. Class II PRA levels: preoperative and 1, 4 months postoperatively. IVIG did not decrease anti-HLA Class II antibody generation.

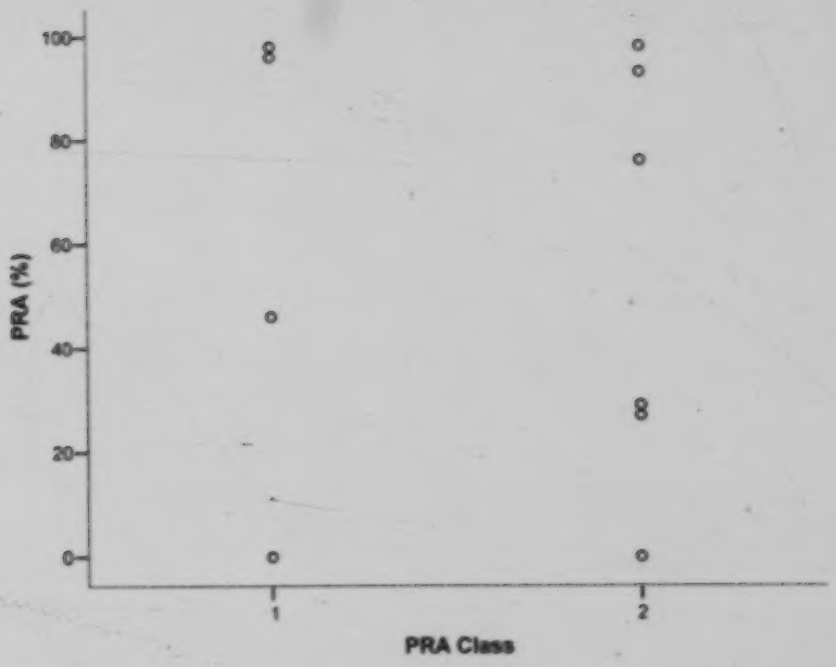

Figure VII-4. Scatter plot of PRA at 4 months after surgery in individual infants receiving IVIG. There was a somewhat dichotomous response to IVIG with responders and non-responders.

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VIII

GENERAL DISCUSION AND CONCLUSIONS

DISCUSSION

The characteristics of the ideal cardiac valve substitute include durability, excellent hemodynamics, resistance to infection, absence of thrombogenicity, and the capacity to remodel, repair, and possibly even grow.[1] The aortic valve allograft, introduced for replacement of the aortic valve in 1962[2] and for reconstruction of the right ventricular outflow tract in 1966[3] most closely approaches these criteria. Despite advancements in bioprosthetic (porcine and bovine) and mechanical valve technology, these devices still lack many of the properties of the ideal valve substitute.[4,5] Moreover, these latter devices lack two more additional criteria: handling properties and hemostasis. Nowhere are these latter two criteria more essential than in congenital cardiac surgery where these tissues are used intact as valved conduits to reconstruct congenitally abnormal valves and used in part (as patches) to reconstruct stenotic structures.

Since they were first introduced, it has been apparent that having allograft tissues readily available was essential and various methods were investigated to sterilize, preserve, and bank these tissues. Early methods of preservation were extremely harsh and, not unexpectedly, the valves failed with predictably rapid time course.[6-8] Realizing that the cellular elements are essential for maintaining the integrity of the native valve through remodeling and repair of the extracellular matrix (ECM) efforts subsequently focused on methods to optimally preserve the cellular viability of these tissues. Advancements in cryopreservation technology allowed for the tissues to be stored in a

such a manner that viability of cellular elements was maintained and durability improved.[9] Others investigated storage in antibiotic solutions for up to 6 weeks prior to implantation.[10] Finally, some groups, in an effort to use valves with maximum cellular viability, promoted the use of fresh ("homovital") valves which had been harvested within 48 hours of implantation.[11]

Despite improvements in outcomes, the allograft valve failed to fulfill the important criteria of durability. In fact, allograft valves have durability which is similar to that of bioprosthetic valves.[12,13] In order to improve the outcomes a paradigm shift was required. Studies of explanted valves revealed an absence of viable cells within one year of implantation.[14] These findings occurred despite "optimal" preservation techniques. Another important study correlated increased donor ischemia times (and presumably reduced viability) with improved clinical outcomes.[15] The conclusion from this study was that enhanced viability was associated with increased antigenicity, suggesting an immunologic mechanism of failure. Thus, paradoxically, the cells which so many investigators have attempted to preserve were causing allograft valve failure through an immune-mediated mechanism. In retrospect, this conclusion is quite logical given that these are allograft tissues being implanted in the absence of immunosuppression. Numerous studies in animal and humans have been discussed in the preceding chapters which confirmed the role of the cellular immune response in allograft failure.[16,17] The role of the humoral immune system, however, was less well understood.

Noted above was the critical role that allograft tissue plays in congenital cardiac surgery. As advancements have been in neonatal resuscitation, surgical techniques, and perioperative care, infants with increasingly complex congenital abnormalities are being

advanced for and surviving surgery. Despite increasing perioperative successes, many children still have residual abnormalities which are not conducive with long-term survival.[18] Consequently, these children are presenting for cardiac transplantation. It has been well documented that preformed anti-HLA antibodies (panel reactive antibodies; PRA) portend poor short- and long-term outcomes in cardiac transplantation.[19] There has been increasing concern that the allograft tissue which is essential for many of the procedures performed would be stimulating the generation of anti-HLA antibodies which may complicate or preclude future transplantation if ever required. Although a number of case series had provided evidence in this regard, most studies were retrospective and lacked a well-matched control group.[20]

In Chapter II we performed a prospective cohort study comparing the PRA of infants undergoing the Norwood procedure for hypoplastic left heart syndrome (HLHS) and receiving an allograft patch to a similar group of infant undergoing the arterial switch procedure for transposition of the great arteries and not receiving allograft. This study clearly demonstrated that cryopreserved allograft tissue used in congenital cardiac surgery is associated with significant sensitization with PRAs approaching 100%. Because of the prospective nature of this study we were able to determine the HLA type of donors and recipients. Moreover, we were able to determine the HLA specificity of the antibodies generated by the recipient. This resulted in two additional findings which confirmed that the allograft was the source of sensitization: the majority of the antibodies generated were specific for the donor's HLA type or respective cross-reactive antigens (CREGS) and in individuals who had the same HLA as the donor (i.e. no HLA mismatch) there was a failure to generate panel reactive antibodies.

Thus, it is now clear that allograft tissue stimulates both cellular and humoral immune responses. While the correlation between the cellular immune response and allograft valve failure is clear, the role of the humoral immune response in this regard is less clear. Even though evidence has been accumulating that the humoral immune response may play some role in allograft valve failure[21] the additional major concern in this regard is the impact of preformed anti-HLA antibodies on future organ transplantation. In a paradigm shift away from preserving allograft cellular viability, removal of all intact cellular elements (decellularization) has been proposed to reduce the immunogenicity of these tissues. By reducing the immunogenicity of these tissues a number of objectives may be achieved: 1) by preventing immune mediated damage to the ECM, it is hypothesized that one would be able to create a structure which is capable of repopulation by host cells which in turn would capable of repair, remodeling, and possibly even growth of the allograft valve; again, important criteria for the ideal cardiac valve substitute, and 2) preventing sensitization which may complicate future transplantation. A preserved ECM would be essential for the former objective to occur as would reducing the immunogenicity of the allograft sufficiently to prevent immune mediated destruction, indeed a fine balance. The methods of decellularization thus mandate a delicate balance between removal of cellular and antigenic material while simultaneously preserving the ECM.

The primary goal of our experiments was to study the immunology of decellularized allograft tissue. Realizing the utility of genetically identical inbred strains of rats for studying the immunology of allograft failure,[16] we chose this model to study the immunology of decellularized aortic valve allografts. Numerous decellularization

protocols have been described for various tissues,[22-24] typically in large animal species; however, there was a paucity of data in small animals. Thus, the goal of Chapter III was to compare a number of previously described decellularization techniques in order to identify an optimal technique for use in a rodent model. We compared three main techniques: osmotic lysis of cells combined with a weak detergent (Triton X-100) to solubilize membranes, osmotic lysis alone, and a trypsin/EDTA based protocol. The trypsin-based protocol was very effective at removing cellular elements but caused extensive destruction of the ECM and thus was considered unsuitable for the purposes of our subsequent studies. The former two protocols were relatively comparable. The osmotic lysis plus detergent protocol (i.e. Triton protocol) was significantly more effective at removing interstitial cellular elements (reduced anti-vimentin staining) and reducing anti-MHC staining compared to osmotic lysis alone when assessed in vitro. When implanted in the infrarenal aorta of syngeneic and allogeneic rats, the Triton protocol was associated with a modest reduction in T cell infiltrates (anti-CD3) compared to osmotic lysis alone. Movat's pentachrome was used in an attempt to assess overall ECM structure but was relatively insensitive due to the lack of a trilaminar structure of valve leaflets in contrast to the leaflets of larger animals including humans. Hydroxyproline assays were used in to approximate ECM content. Interestingly, the Triton protocol was associated with an increase in hydroxyproline content per dry weight compared to osmotic lysis alone and to control (nondecellularized) tissue, suggesting that this technique caused increased solubilization of nonfibrillar ECM elements such as glycosaminoglycans (GAGs). Thus, the Triton protocol was associated with reduced antigenicity in vitro and reduced immunogenicity in vivo but was slightly more toxic to

the ECM as compared to osmotic lysis alone. While these findings represent competing goals of decellularization, it was felt that the additional reduction in immunogenicity with low dose Triton X-100 was essential for preventing long term immune-mediated damage and this protocol would be the focus of subsequent investigations.

Because of the concerns about potential long-term toxicity caused by residual Triton X-100 in the allograft valves, studies were performed to determine the residual amount of Triton X-100 present after decellularization and whether the protocol required revision. In Chapter IV, rat aortic valves which had been decellularized using the Triton protocol were allowed to equilibrate for a fixed period of time in distilled water after washouts in phosphate buffered saline of varying durations. ^{1}H NMR was then used to detect residual amounts of Triton X-100 in the distilled water. This study demonstrated that the majority of Triton X-100 was washed out within 4 hours, that there was minimal release of Triton X-100 from the valve after 4 hours, and the washout period in PBS may be shortened from 72 hours to less than 24 hours if necessary. Importantly, this study demonstrated that Triton X-100 is safe.

The primary reason for decellularizing tissue is to reduce its immunogenicity. Chapter V assessed the cellular and humoral immune response to decellularized aortic valve allografts in an in vivo rat model. Decellularized and nondecellularized (fresh) control aortic valves were implanted into the infrarenal aorta of syngeneic (Lewis-Lewis) and allogeneic (Brown Norway-Lewis) rats. Valves were explanted at 1, 2, 4, 16 weeks; serum was harvested at 2, 4, 16 weeks. T cell infiltrates, identified with immunohistochemistry for CD3+ and CD8+ cells and quantified with morphometric analysis software, identified significant reductions in cellular infiltrates in decellularized

allograft tissues. T cell infiltrates were reduced to levels similar to that of syngeneic tissues. Flow cytometric analysis using Brown-Norway splenocytes as target cells and goat anti-rat FITC labeled secondary antibody demonstrated complete abrogation of the humoral immune response to decellularized allograft tissue. These are extremely important findings: abrogation of the cellular immune response may improve long-term durability and abrogation of the humoral response could positively impact subsequent solid organ transplantation. The weakness of this study was the formation of thrombus in the sinus of Valsalva of decellularized grafts. This was likely the result of the combination of the nonworking nature of this model and the removal of the endothelial layer by the decellularization process. Additional studies in larger animals in a position in which the leaflets have an opportunity to open and close during the cardiac cycle will be essential to assess this further.

The ultimate goal of our research is to develop a decellularized allograft for implantation into humans. Before that goal can be reached the second objective of decellularization must be confirmed: preservation of the ECM and the biomechanical properties of the tissue. To that end, in Chapter VI we performed biomechanical testing of decellularized tissue. Due to size limitations, such testing in rodent tissue was not possible. Porcine tissue is readily available, closely approximates the anatomy and histology of human aortic valve allografts, and is of a size which is suitable for biomechanical testing. Strips of porcine ascending aorta and pulmonary artery were decellularized and compared to nondecellularized tissues. Static testing using an MTS Instron 500 revealed similar stress-strain curves and stress at fracture for decellularized and nondecellularized tissues. The elastic modulus was calculated from the slope of the

stress-strain curves and no significant differences were identified between the treatment and control groups. These in vitro studies suggest preservation of the biomechanical properties of decellularized tissues.

Additional research and development will be required before decellularized tissue can be used clinically. During this time period infants will continue to receive allograft tissue and will continue to become sensitized to this tissue, potentially complicating future transplantation. Although commonly used immunosuppressives are effective, their use can not be justified due to toxicity. Intravenous immunoglobulin (IVIG) is an agent which is safe, has multiple immunomodulatory effects, and has been shown to effectively desensitize individuals with elevated PRAs awaiting organ transplantation. It was hypothesized that IVIG could prevent sensitization of infants receiving allograft tissue and thus would provide a safe alternative until decellularized tissues had undergone the necessary development. In Chapter VII, a pilot study with seven infants receiving allograft tissue, IVIG (2 g/kg) was given prior to surgery and 3, 16 weeks after surgery. Despite documented efficacy in reducing PRAs in individuals who had been previously sensitized, IVIG was not effective in preventing sensitization. A major limitation of this study was the need to administer the IVIG while in hospital and the consequent inability to administer IVIG at 2 and 3 months after surgery when the child was most likely developing memory B cells to the allograft tissue.

The ultimate goal will be to translate our findings into a clinically useful device. Recognizing the substantive differences between rodents and humans, ongoing studies will be required to assess these tissues before such a goal can be achieved. Sheep have been the classic model for assessment of valve durability and we are currently in the

process of reproducing our work in this larger animal model. It will be essential to confirm in this larger model that decellularization reduces immunogenicity and preserves the biomechanical properties of the tissue. This model would also allow for further assessment of the potential of these tissues for repopulation by host cells, in turn creating a permanent, nonthrombogenic valve substitute which will have the capacity for remodeling, repair, and possibly even growth. Additional issues which will need to addressed include the necessity for in vitro reseeding of the grafts prior to implantation (vs. in vivo seeding), the method of in vitro reseeding (e.g. pulse duplicator), and the cell types for reseeding (endothelial cells, vs. fibroblasts, vs. mesenchymal stem cells). Lastly, because these tissues will need to be banked for extended time periods, the impact of cryopreservation on decellularized tissues will need further investigation.

CONCLUSIONS

The experiments included in this thesis have made significant advancements in the field of decellularized allograft tissue used in cardiac surgery. Significant findings include:

I. the use of cryopreserved allograft tissue in congenital cardiac surgery is associated with a significant donor-specific humoral response in the majority of patients, especially in those who were mismatched for HLA type,

II. this sensitization may increase the risk of early graft failure and poorer patient survival if subsequent organ transplantation is required,

III. alternatives tissues such as an autologous pericardial patches or methods such as decellularization to make the allograft tissue less immunogenic should be considered,

IV. that decellularization of rat aortic valve allografts with a combination of hypotonic and hypertonic buffers, Triton X-100, endonucleases, and washout in PBS most effectively balances the goals of decellularization: reduction of antigenicity and preservation of ECM,

V. that the decellularization protocols which use Triton X-100 are safe and are associated with minimal residual Triton X-100 leachate from the decellularized tissue,

VI. that a minimal final wash time of four hours is required to reduce Triton X-100 to non-toxic levels for rat aortic valves,

VII. that decellularization of aortic valve allografts is associated with a significant reduction in cellular and humoral immune responses to levels seen with nonimmunogenic syngeneic tissue,

VIII. that decellularization may protect the allograft tissue from immune-mediated damage, in turn increasing allograft durability and creating an acellular matrix that may be a suitable environment for repopulation by host cells (e.g. fibroblasts) which would provide the extracellular matrix with a regenerative capacity typical of native valves,

IX. that decellularization, through a reduced humoral response, may prevent sensitization of recipients of allograft tissue. This would have enormous implications for the survival and quality of life of all valve recipients, especially those who may require subsequent organ transplantation,

X. that static testing of decellularized porcine ascending aorta and pulmonary artery indicates that decellularization does not negatively impact the biomechanical properties of these tissues,

XI. that despite studies in adults which have demonstrated that high dose (2 g/kg) intravenous immunoglobulin (IVIG) reduces sensitization, IVIG does not demonstrate benefit when given to prevent sensitization,

XII. that ongoing studies in a larger animal model of orthotopic implantation will provide information on the thrombogenicity, functional integrity, and capacity for repopulation by host cells.

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